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**(54) Title:** PRODUCTION OF RECOMBINANT PROCOLLAGEN IN YEAST

**(57) Abstract**

The invention discloses methods for effecting the production of recombinant mammalian procollagen in yeast, as well as compositions comprising recombinant yeast cells capable of producing mammalian procollagen.

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*PRODUCTION OF RECOMBINANT PROCOLLAGEN IN YEAST*INTRODUCTIONField of the Invention

The field of this invention is the production of recombinant procollagen in yeast.

5 Background

Collagen has provided a number of commercial applications, including use as a convenient substrate for cell culture, as well as in the manufacture of biocompatible materials (e.g., artificial skin) having a variety of therapeutic applications in humans. Commercially available collagens are primarily isolated from freshly killed farm animals (e.g., cows, pigs, chickens) in polymerized form or in soluble form derived by enzymatic or chemical treatment of polymerized collagen. However, polymerized collagens, and soluble collagens derived from polymerized collagens, are of limited usefulness due to the presence of natural crosslinks within the collagen. Furthermore, non-human collagens can provoke undesirable immune responses when administered to human subjects.

15 As one alternative, human collagen can be purified from human sources such as human placenta, as described in U.S. Patent No. 5,002,071 (Research Development Foundation) and in copending U.S. Patent No. 5,428,022 (Collagen Corporation). In addition to source limitations and the risk of contamination by human pathogens such as Hepatitis viruses and HIV, the methods for recovering collagen from these sources bias the type of collagen recovered. Furthermore, because the collagen is derived from naturally crosslinked tissues, the collagen recovered is not entirely homogeneous. Another approach to the production of 20 human collagen is the expression of recombinant human collagen in the milk of transgenic animals, as described in PCT publication WO 9416570 (Collagen Corporation). However, this approach subjects the recombinant collagen to any host deficiencies in translational 25 processing.

Collagen polypeptide chains are encoded by a number of related genes. See S. L. Adams, *Amer. J. Respir. Cell and Mol. Biol.* (1989) 1:161-168; Mayne and Brewton, *Current Opinion in Cell Biology* (1993) 5:883-890; and van der Rest and Bruckner, *Current Opinion in Structural Biology* (1993) 3:430-436. Collagen-encoding transcripts are initially translated into procollagen chains which undergo a variety of post-translational events such as processing, secretion, and assembly (e.g., disulfide exchange, prolyl residue hydroxylation,

lysyl residue hydroxylation, glycosylation, assembly into trimeric molecules, and crosslinking of the helical chains; see, for example, Prockop et al., *New England J. Med.* (1984) 311:376-386) to form mature collagen chain helices. In addition, procollagens contain N- and C-terminal propeptides that facilitate the assembly of trimeric collagen molecules and helix formation. See, for example, Lee et al., *J. Biol. Chem.* (1992) 267: 24126-24133.

5 It is these unique properties of collagen, including the protein size and structure, the requisite post-translational processing, the secretory pathway, the nature of the collagen transcript and gene itself and its assembly into trimeric molecules, etc., that have severely restricted the ability of *in vitro* cellular expression systems to produce recombinant collagen or 10 procollagen.

#### Relevant Literature

For reviews, see Nothwehr et al., *J. Biol. Chem.* (1994) 269:10185-10188, regarding the sorting of membrane proteins in the yeast secretory pathway; Gellissen et al. *van Leeuwenhoek* (1992) 62:79-93 regarding heterologous protein production in yeast; and 15 Hitzeman et al., *Meth. Enzymology* (1990) 185:421-441 regarding the use of secretion signal sequences for secretion of heterologous proteins from yeast.

Heterologous proteins which have been reportedly expressed in yeast include 20 hemoglobins (Mould et al., *Biochem. J.* (1994) 298:619-62; Wagenbach et al., *Bio/technology* (1991) 9:57-61); a mammalian ATPase (Horowitz et al., *J. Biol. Chem.* (1990) 265:4189-4192); and human lipocortin I (Giga-Hama et al., *Bio/technology* (1994) 12:400-404).

Robinson et al., *Bio/technology* (1994) 12:381-384, describe the use of protein disulfide isomerase overexpression to increase secretion of foreign proteins in yeast.

#### SUMMARY OF THE INVENTION

25 The invention provides methods and compositions for making mammalian procollagen in yeast. Generally, the methods of the invention comprise: (a) incubating a recombinant yeast cell comprising (i) a stable genetic construct comprising a mammalian collagen gene comprising a secretion signal sequence and a promoter heterologous to said collagen gene, the collagen gene being operably linked to the promoter, in a medium under conditions wherein 30 the collagen gene is expressed as a procollagen chain, and the procollagen chain is secreted through the plasma membrane and released from the cell wall of the yeast into the medium in

the form of triple helical procollagen molecules; and (b) recovering the resulting triple helical procollagen. According to preferred embodiments, the secretion signal sequence is heterologous to the collagen gene; substantially all of the procollagen chains expressed by the yeast cell are secreted through the plasma membrane and released from the cell wall of the 5 yeast; the yeast cell further comprises (ii) a prolyl hydroxylase gene and (iii) a protein disulfide isomerase gene, and each of the prolyl hydroxylase gene and the protein disulfide isomerase gene is expressed as an active protein during the incubating step; the construct further comprises a selectable marker, such as an invertase gene, *LEU2(d)*, *TRP1*, *URA3*, *HIS3*, or a combination thereof; and the yeast cell is *Saccharomyces cerevisiae*.

10 The compositions of the invention comprise recombinant yeast cells capable of producing mammalian procollagen. Such cells comprise: (i) a stable genetic construct comprising a mammalian collagen gene comprising a secretion signal sequence and a promoter heterologous to the collagen gene, wherein the collagen gene is operably linked to the promoter. According to preferred embodiments, the secretion signal sequence is heterologous 15 to the collagen gene; the recombinant yeast cell further comprises (ii) a prolyl hydroxylase gene and (iii) a protein disulfide isomerase gene; the construct further comprises a selectable marker, such as an invertase gene, *LEU2(d)*, *TRP1*, *URA3*, *HIS3*, or a combination thereof; and the yeast cell is *Saccharomyces cerevisiae*.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 and 2 illustrate the structure of plasmids Gp5012 and Gp5020, respectively.

Figures 3 and 4 illustrate the structure of plasmids Gp5105 and Gp5106, respectively.

Figures 5 and 6 illustrate the structure of plasmids GpGp5111 and Gp5112, respectively.

Figure 7 illustrates the structure of plasmid Gp5098.

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Figures 8 and 9 illustrate the structure of plasmids Gp5099 and Gp5100, respectively.

Figures 10 and 11 illustrate the structure of plasmids Gp5101 and Gp5102, respectively.

Figures 12 and 13 illustrate the structure of plasmids Gp5096 and Gp5097, respectively.

Figures 14 and 15 illustrate the structure of plasmids Gp5107 and Gp5108, respectively.

Figures 16 and 17 illustrate the structure of plasmids Gp5113 and Gp5114, respectively.

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Figure 18 illustrates the rate of increase in O.D.600 of transformed *Saccharomyces cerevisiae* cultures grown at 20°C for several days. Each transformed *S. cerevisiae* culture

harbors a multicopy plasmid containing a different leader sequence linked to the human  $\alpha 1(I)$  procollagen gene. The control sample is a transformed *S. cerevisiae* culture which contains a multicopy plasmid without the human  $\alpha 1(I)$  procollagen gene.

Figure 19 illustrates the structure of plasmid Gp5091.

5 Figure 20 illustrates the structure of plasmid Gp5220.

Figures 21 and 22 illustrate the structure of plasmids Gp5217 and Gp5218, respectively.

Figures 23 and 24 illustrate the structure of plasmids Gp5219 and Gp5220, respectively.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

10 The invention provides a breakthrough in the ability to produce recombinant mammalian collagen; in particular, in cultured yeast cells. The subject methods and compositions are used to produce commercially useful amounts of functional human collagen at low production costs. The term collagen refers to a type of protein that encompasses a class of structurally related polypeptides consisting of homo- and hetero- polymers, generally 15 trimeric polymers, of collagen chains. The pyrrolidone rings in the  $\alpha$ -amino acids of each chain are on the outside of the triple helix; the fixed angle of the C-N peptidyl bonds enable each collagen chain to fold into a helix with a geometry such that three chains twist together to form a three-stranded helix. Collagen chains comprise of (GXY)n- repeats and are capable of forming collagen such that there are three amino acids per turn of the collagen helix, every 20 third residue is glycine and the side chains of the glycines are positioned inside the helix. The repeat regions of the chains are rich in proline and hydroxyproline, where the X position is preferably occupied predominantly by proline or hydroxyproline, preferably proline. The chains preferably also comprise non-helical extensions, or telopeptides, which facilitate interchain polymerization. Hence, collagen chains encompass natural gene products as well 25 as mutant and synthetic gene products having the requisite collagen chain structure.

The general methods of the invention involve incubating a recombinant yeast cell comprising a stable genetic construct, which may be integrated into the yeast genome, or maintained on a mini-chromosome or stable episomal plasmid within the yeast cell to maximize copy number and expression yield. The construct comprises a mammalian 30 collagen gene comprising a secretion signal sequence and a promoter heterologous to the collagen gene, wherein the collagen gene is operably linked to the promoter. The yeast cell is incubated in a medium under conditions wherein the collagen gene is expressed as a

procollagen chain, which is secreted through the plasma membrane and cell wall of the yeast into the medium in the form of triple helical procollagen. Finally, the expressed triple helical procollagen is recovered from the medium. The collagen gene, secretion signal sequence, promoter, yeast, and incubation conditions are selected such that substantially all of the 5 procollagen chains expressed by the yeast cell are secreted through the plasma membrane and released from the cell wall of the yeast.

The collagen gene used is selected from the closely related collagen gene family including  $\alpha 1$ -2(I),  $\alpha 1$ (II),  $\alpha 1$ (III),  $\alpha 1$ -6(IV),  $\alpha 1$ -3(V),  $\alpha 1$ -3(VI),  $\alpha 1$ (VII),  $\alpha 1$ (XIII),  $\alpha 1$ -3(IX), 10  $\alpha 1$ (X),  $\alpha 1$ -3(XI), and  $\alpha 1$ (XII). The basic structure, amino acid composition, and codon usage of these genes are very similar and generally permit ready substitution of one collagen gene for another in the subject methods. See, for example, S. L. Adams, *Amer. J. Respir. Cell and Mol. Biol.* (1989) 1:161-168; Mayne and Brewton, *Current Opinion in Cell Biology* (1993) 5:883-890; and van der Rest and Bruckner, *Current Opinion in Structural Biology* (1993) 3:430-436.

15 To produce heterologous collagens comprising different collagen chains (e.g., type I collagen comprises a heterotrimer consisting of two  $\alpha 1$ (I) chains and an  $\alpha 2$ (I) chain), the methods provide for the co-introduction of all requisite collagen genes into the host yeast cell for simultaneous expression. Association of the various collagen chains into multi-chain units is controlled by the relative supply of each chain.

20 The collagen structural gene requires a secretion signal sequence capable of directing nascent collagen chains through a secretory pathway in order to effect efficient secretion of the collagen from the yeast into the periplasmic space. In the disclosed expression system, most, preferably essentially all, of the procollagen translated is secreted into the periplasmic space and much, preferably most and, more preferably, essentially all, of the secreted procollagen is secreted through the yeast cell wall as well. We have found several 25 heterologous secretion signal sequences that resulted in the secretion of a substantial amount of the expressed procollagen through the cell wall. In particular, we identified the yeast  $\alpha$  factor prepro sequence and the human HSA prepro sequence as providing secretion signal sequences capable of effecting the secretion of substantially all of the expressed procollagen. 30 Additional suitable secretion signal sequences are identified empirically, as described in Example 2, below.

The collagen gene and secretion signal sequence are operably linked to a promoter heterologous to the collagen. A wide variety of constitutive and inducible promoters can be used. In particular, we found the following heterologous promoters well-suited to the transcriptional regulation necessary to practice the subject methods (see Romano et al., *Yeast* 5 (1992) 8:423-488):

GAL1-10	CHELATIN	PGK	GAP	TPI
ADH <sup>2</sup> PHOS	MF $\alpha$ 1	MF $\alpha$ 2	PGK/ $\alpha$ 2*	TPI/ $\alpha$ 2*
GAP/GAL*	PGK/GAL*	GAP/ADH <sup>2</sup> *	GAP/PHOS*	CYC1/GRE*
PGK/ARE*				

10 \* Denotes a hybrid promoter.

The host yeast cell provides a means or marker to select for recombinant cells which have been stably transformed with the collagen construct. Essentially any convenient means or marker that provides a convenient means for positive or negative selection may be used. Generally, the means or marker is a gene introduced into the host cell which confers on the 15 cell a selective growth or survival advantage in one or more environments. Some examples of suitable selectable markers include an invertase gene, *LEU2(d)*, *TRP1*, *URA3*, and *HIS3*. Alternatively, the expressed procollagen itself can provide selective means. For example, automated fluorescent activated cell sorting (FACS) is conveniently used to segregate 20 procollagen-expressing cells labeled with fluorescein-labeled anti-collagen antibody. In addition, procollagen-expressing cells can be selected by secondary phenotype alterations caused by procollagen expression, such as an increased tendency for cells to aggregate, as described in Example 5, below.

Any convenient yeast host cell may be used in the subject methods. For example, well-established transfection and expression systems are available for *Saccharomyces cerevisiae*, *Saccharomyces pombe*, and *Pichia pastoris*. In a preferred embodiment, *Saccharomyces cerevisiae* is used. In addition to the collagen construct and selectable marker, the recombinant yeast cells may also comprise additional genetic elements to improve yield and/or provide additional post-translational processing. For example, in one embodiment, the yeast cell further comprises (ii) a prolyl hydroxylase gene and (iii) a protein disulfide isomerase gene. The yeast may also express an exogenous site-specific proteolytic agent capable of specifically cleaving the procollagen at the propeptide recognition site to provide mature collagen.

In one specific embodiment of the invention, the host yeast cells have one or more mutations that cause the cell wall of the yeast cell (which is primarily composed of the polysaccharides chitin and glucan) to exhibit increased permeability to high molecular weight proteins, such as procollagen. The increased permeability of the cell wall results in the 5 release from the cell wall of an increased proportion of the triple helical procollagen produced in the yeast cell. Typically, these mutations are in the genes that code for various enzymes (such as chitin synthetase and glucan synthetase) required for the synthesis of the major polysaccharide components of the cell wall, or in the genes that code for enzymes required to effect the N-glycosylation or O-glycosylation of the glycoprotein components of 10 the cell wall. In this embodiment, we find that procollagen expression can enhance the stability of the genetically defective walls, providing another means for selection. For example, the cells can be grown under stress conditions which provide procollagen-expressing cells preferential growth or survival. Suitable stress conditions for such selection include the presence of denaturing reagents such as detergents (e.g., 0.005% SDS), urea, etc.

15 A specific embodiment of the invention is defined by low temperature incubation conditions, which we found to be important for efficient expression of triple helical procollagen in yeast when prolyl hydroxylase is not present. In particular, we found that efficient expression of triple helical procollagen, in the absence of prolyl hydroxylase, occurs at incubation temperatures between about 15°C and about 25°C, preferably at about 20°C. In 20 addition, to facilitate efficient expression at these reduced temperatures, we found that the conventional minimal media used in yeast expression systems should be augmented with supplemental amino acids. When prolyl hydroxylase is present, higher incubation temperatures may be required, the optimal incubation temperature being dependent on the rate of triple helix formation.

25 Procollagen and/or collagen chains are purified from the medium by methods known in the art. See, for example, Miller and Rhodes, *Methods in Enzymology* (1982) 82:33-64, or Sage and Bornstein, *Methods in Enzymology* (1982) 82:96-127. Resulting collagen compositions may be sterilized, for example, by passage through 0.2  $\mu\text{m}$  filters (e.g., Millipore filters). Sterility may be confirmed by, for example, culturing aliquots on nutrient 30 agar for at least 72 hours at 25 and 37°C. For therapeutic uses, it is desirable to ensure the non-toxicity, biocompatibility, and non-immunogenicity of the resulting recombinant

collagen compositions. For example, non-immunogenicity in humans is confirmed by dermal sensitivity testing.

The invention provides a wide variety of applications of the subject recombinant procollagens and collagens in tissue and cell culture and therapy. For example, the subject procollagens and collagens are used to promote adhesion and growth of cells on a wide variety of solid substrates in various forms, such as slides, filaments, sheets, plates, flasks, bottles, fibers, etc., and compositions such as plastics, glass, metals, saccharide-based polymers, etc. The collagens provide a wide range of therapeutic applications, such as use in biodegradable sutures, synthetic dermal skin, biodegradable hemostatic sponges, tissue augmentation (including modifying localized soft tissue appearance), etc.

Tissue augmentation using collagen compositions has been thoroughly studied, documented, and implemented in widespread clinical settings. See, for example, DeLustro et al., *J. Biomed. Mater. Res.* (1986) 20:109-120; Elson, *J. Dermatol. Surg. Oncol.* (1989) 15:301-303; Elson, *Am. J. Cosmetic Surg.* (1992) 9:267-271; Klein et al., *J. Dermatol. Surg. Oncol.* (1985) 11:337-339; Klein et al., Geriatric Dermatology: Clinical Diagnosis and Practical Therapy, Chapter 8, pp. 47-49, Igaku-Shoin (publ), NY (1989); Kligman, *J. Dermatol. Surg. Oncol.* (1988) 14 (suppl I) :35-38; Kligman et al., *J. Dermatol. Surg. Oncol.* (1986) 12:351-357; Kligman et al., *J. Dermatol. Surg. Oncol.* (1988) 14 (suppl I) :10-12; Knapp et al., *Plas. Reconstr. Surg.* (1977) 60:398-405; Knapp et al., *J. Surg. Res.* (1977) 23:96-105; Varnavides et al., *Br. J. Dermatol.* (1987) 116:199-206; etc.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

25

### Example 1

#### (Native Procollagen Yeast Expression Vector)

The human  $\alpha 1(I)$  procollagen cDNA is described in A. Stacy, R. Mulligan, R. Jaenisch, *J. Virol.* (1987) 61:2549-2554. The human  $\alpha 1(I)$  procollagen cDNA was cloned into Bluescript II between the *Eco*RI and *Ssp*I sites (Stratagene Cloning Systems, La Jolla, CA), generating pDT2025 and, from this vector, recloned either between the *Sa*II and *Sna*BI sites of pSV-SPORT1 (Gibco-BRL, Gaithersburg, MD), generating construct pDT2067, or between the *Hind*III and *Eco*RV sites of pcDNA3 (Invitrogen Corporation, San Diego, CA),

yielding construct pDT2068. The 4550 bp *Xba*I fragment from pDT2068 was cloned into yeast expression vectors Gp5012 and Gp5020 (see Figure 1) using the *Nhe*I site in the polylinkers. Plasmids Gp5105 and Gp5106 (see Figure 2) resulted from this attempt.

It should be noted that the structure of the 3' end of the construct shown in Figure 2 is different from that in Gp5101 and Gp5102 (see Figure 6, described in Example 2, below). A number of extra sites, including a second *Not*I site, have been introduced by the inclusion of a small portion of the polylinker from the pDT2068, from which the *Xba*I fragment was isolated. To work on yield improvement, it would be desirable to only have the one unique *Not*I site located 5' to the yeast promoter at coordinate 5057 bp in Gp5105 map (see Figure 2). To accomplish this, we isolated the *Not*I - *Kpn*I fragments from Gp5105 (620 bp) and Gp5106 (853 bp) containing the yeast promoter/collagen 5' end junction and cloned them into *Not*I - *Kpn*I cut Gp5101 (see Figure 6) to create Gp5111 and Gp5112 (see Figure 3). These plasmids now have the native collagen secretion signal with the same 3' junction structure as the other expression constructs described in Example 2. They also now have only the one *Not*I site near the promoter element. All four of the native secretion signal constructs have been transformed into the following yeast strains:

GY5196      MAT  $\alpha$  *leu2*- $\Delta$ 1 *trp1*- $\Delta$ 63 *ura3*-52 *his3*- $\Delta$ 200 GAL

GY5198      MAT  $\alpha$  *trp1*- $\Delta$ 63 *ura3*-52 GAL

GY5196 and GY5198 were obtained from a genetic cross of X2180-1B (Yeast Genetic Stock Center Catalog, U.C. Berkeley, 7th ed., 1991) and YPH499 (Sikorski and Hieler, *Genetics* (1989) 112:19-27). Selection for Trp+ complementation (Current Protocols in Molecular Biology, Kaaren Janssen, Ed., Chapter 13, John Wiley & Sons, USA) has yielded hundreds of transformants for each plasmid.

#### Example 2

25            (Procollagen Yeast Expression Vector With Altered Secretion Signal)

Altering the 5' end of the  $\alpha$ (I) collagen coding region to remove the native secretion signal would allow us to add various heterologous and homologous secretion signals which previously have worked well in yeast. Oligonucleotide primers were used in the PCR™ reaction (H. A. Erlich, D. Gelfand, J. J. Sninsky, *Science* (1991) 252:1643-1651) to synthesize a DNA fragment corresponding to the coding region of the amino-propeptide and the first 17 amino acids of the triple helical region of human  $\alpha$ 1(I) procollagen with the addition of *Eco*RI/*Sal*II and *Bgl*II/*Hind*III restriction sites at the 5' and 3' ends, respectively,

of this DNA fragment. The product was cloned into vector pUC118 at the *Eco*RI and *Hind*III sites for sequencing (see Figure 4). We cloned the PCR product from the above plasmid into our expression vectors containing the prepro-HSA secretion signal (R. Hitzeman et al., *Meth. Enzymol.* (1990) 185: 421-440) using the *Bg*II and *Sal*I sites (see Figure 4), 5 generating plasmids Gp5099 and Gp5100 respectively (see Figure 5). We then added the rest of the  $\alpha$ (I) procollagen coding region by ligating the 4389 bp *Kpn*I - *Ssp*I fragment from pDT2025 to *Kpn*I - *Pvu*II digested vectors Gp5099 and Gp5100 to generate plasmids Gp5101 and Gp5102 respectively (see Figure 6).

We have also constructed plasmids that contain the yeast  $\alpha$  factor prepro sequence 10 joined to the  $\alpha$ (I) procollagen coding region. The first step was to clone the 525 bp *Bg*II - *Sal*I  $\alpha$ (I) collagen PCR fragment (see Figure 4) into *Bg*II - *Sal*I cut vectors Gp5096 and Gp5097 (see Figure 7) to generate plasmids Gp5107 and Gp5108 (see Figure 8). Vectors Gp5096 and Gp5097 contain the yeast chromosomal  $\alpha$  factor prepro sequence isolated by 15 PCR from yeast strain 20B-12 (Jones, *Genetics* (1977) 85:23-33) and inserted into (Kurjan and Herskowitz, *Cell* (1982) 30:933-943) our expression vector with either the chelatin (Etcheverry, *Meth. Enzymol.* (1990) 185:319-327) or galactose 1-10 (Johnson et al., *Cell* 20 (1987) 50:143-146) promoter elements, respectively.

The next step was to clone the *Kpn*I - *Not*I fragments from plasmids Gp5107 and Gp5108 (802 bp and 1035 bp, respectively) into *Kpn*I - *Not*I cut Gp5101 vector to generate 20 expression plasmids Gp5113 and Gp5114 (see Figure 9).

### Example 3

#### (Collagen Expression Studies)

Analysis of yeast extracts by SDS/PAGE gels indicated that there are no major yeast 25 proteins that co-migrate in the region where we would expect  $\alpha$ (I) procollagen to be on either 5% or 8% SDS:PAGE gels (U. K. Laemmli, *Nature* (1970) 227:680-685). A 'collagenase' activity was identified in the periplasmic preparations of yeast extracts. The 'collagenase' 30 activity appears to be derived from the enzyme preparation (Zymolyase T100, ICN Biochemicals) used to lyse the yeast cell wall and release the periplasmic proteins. We found that this activity seemed to be processing the procollagen to collagen and could be inhibited by PMSF, indicating the involvement of a serine protease.

Yeast transformants were grown for 72 hours at 10°, 20°, 25°, or 30°C in selective medium in the presence or absence of inducer (0.2 mM copper sulfate for the chelatin

promoter and 2% galactose for the gal1-10 promoter). Twenty O.D. unit pellets (equivalent of 20 ml of cells at O.D.600=1.0) were collected and resuspended in 100  $\mu$ l of SDS sample buffer or phosphate-buffered saline (PBS) plus 5 mM EDTA and 1 mM PMSF. Acid-washed glass beads (Sigma, St. Louis, MO) were added and the sample vortexed at room

5 temperature for

7 to 15 minutes. SDS sample buffer was added to samples resuspended in PBS. The samples were incubated at 75°C for 5 minutes and the debris collected by centrifugation. Clarified supernatants were loaded onto 6% SDS:PAGE gels.

The gels were western blotted (H. Towbin, T. Staehelin, and J. Gordon, *Proc. Nat. Acad. Sci.* (1979) 76:4350-4354) onto PVDF membranes (Millipore Corp., Bedford, MA), probed using an antibody against the amino-propeptide region of human  $\alpha$ 1(I) procollagen (LF-39, L. W. Fisher, W. Lindner, M. F. Young, and John. D. Termine, *Connective Tissue Res.* (1989) 21:43-50) and a horseradish peroxidase-linked secondary detection antibody (Kirkegaard and Perry, Gaithersburg, MD), followed by development using the ECL™

10 Detection Kit (Amersham Life Science, Inc., Arlington Heights, IL). Results of the analysis showed the presence of antibody-reactive protein migrating in the expected location for  $\alpha$ 1(I) procollagen. To further confirm that yeast transformed with the human  $\alpha$ 1(I) procollagen

15 cDNA were directing the synthesis of procollagen protein, yeast extract was treated with bacterial collagenase (Bacterial Collagenase Form III, Advance Biofrazures Corp., Lynbrook, NY), which digests the triple helical region of procollagen. The results demonstrated the sensitivity of the antibody-reactive band to this enzyme.

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#### Example 4

##### (Quantification of Procollagen Synthesized in Yeast)

Human  $\alpha$ 1(I) procollagen was isolated from SVWI-38 cells (M. I. Parker, A. A. Smith, K. Mundell, M. Collins, S. Boast, and F. Ramirez, *Nuc. Acids Res.* (1992) 20:5825-5830) and used as a standard to quantify human  $\alpha$ 1(I) procollagen produced by yeast. Both the SVWI-38 derived procollagen and yeast extract were diluted, run on 6% SDS:PAGE, and western blotted using LF-39 antibody. A comparison of relative signal strength between the standard and the procollagen in the yeast extract was used to estimate the amount of

25 procollagen in the yeast extract. The total protein concentration of the yeast extract was determined using the BCA Protein Kit (Pierce, Rockford, IL). Based on the protein assay, the amount of  $\alpha$ 1(I) procollagen produced was approximately 0.3% of the total protein in the

30

yeast extract. Visible bands on silver stains of SDS gels suggest that levels of the collagen may be much higher. Since the antibody recognizes the amino-terminal portion of procollagen, processed forms of procollagen are not detected by the western blotting method.

5 The relative intensities of anti-collagen antibody-reactive material in western blots were compared between yeast transfections using the two different promoters, the three different leader sequences linked to the human  $\alpha 1(I)$  procollagen cDNA, and the two yeast strains. Both yeast promoters produce procollagen. The levels of procollagen produced using the three different secretion signals have the following relationship:

prepro $\alpha$ factor-procollagen > preproHSA-procollagen > preprocollagen.

10

#### Example 5

(Growth Characteristics of Yeast Producing Human Procollagen)

Figure 10 shows growth curves of yeast induced for procollagen production (medias are identical) at 20°C where the only variables are +/- the procollagen gene expression and three different secretion signals on the same human procollagen protein. The native human 15 procollagen signal results in no growth rate difference compared to a nonprocollagen producing strain (same plasmid present, but without the procollagen gene), while the other two secretory signals result in a significant decrease in growth rate (especially with the  $\alpha$  factor prepro signal). The growth, although slowed, does eventually reach the same stationary phase.

20

A visible yeast characteristic is noticed under the microscope in direct relationship with this slow growth: yeast cell clumping (i.e., lack of efficient mother/daughter cell separation after mitosis). This characteristic often coincides with cell wall changes or defects. This clumping is extremely dramatic for yeast producing secreted procollagen using the  $\alpha$  factor prepro sequence.

25

#### Example 6

(Localization of the Human Procollagen Produced in Yeast)

Yeast fractionation experiments were done according to R. Hitzeman et al., *Nuc. Acids Res.* (1983) 11:2745-2763, and *Current Protocols of Molecular Biology*. Yeast containing the human  $\alpha 1(I)$  procollagen cDNA was fractionated into intracellular, 30 periplasmic, and media fractions. Media was concentrated approximately 40-fold using CentriCell 20 centrifugation. Each fraction was electrophoresed on a 6% SDS:PAGE and

western blotted. All the procollagen was found in the extracellular periplasmic and media fractions.

#### Example 7

##### (Trimeric Formation of the Human Procollagen Produced in Yeast)

5 The triple helical region of the procollagen molecule, comprised of three polypeptide chains in the correct orientation, is resistant to endoproteases such as trypsin, chymotrypsin, and pepsin (P. Bruchner and D. J. Prockop, *Anal. Biochem.* (1981) 110:360-368). Mammalian  $\alpha 1(I)$  procollagen triple helical molecules are stable to greater than 37°C due to hydroxylation of proline residues within the triple helical region of the procollagen molecule 10 (A. E. Geddis, D. J. Prockop, *Matrix* (1993) 13:399-405).

To determine whether the human  $\alpha 1(I)$  procollagen polypeptides synthesized in yeast are associating into the correct triple helical structure, yeast extracts containing the 15 preproHSA-procollagen were digested with pepsin (Worthington Biochemical Corp., Freehold, NJ) at increasing temperatures. Using a 6% SDS:PAGE stained with the Silver Stain Plus Kit (Bio-Rad Laboratories, Hercules, CA), we showed that the triple helical region of the  $\alpha 1(I)$  procollagen protein is resistant to pepsin digestion at 20 and 23°C. At higher 20 temperatures, the collagen band disappears, which suggests that the triple helix is dissociating and thereby susceptible to pepsin digestion. Yeast does not synthesize the enzyme necessary to hydroxylate proline residues of procollagens. The melting temperature (Tm) of the triple helix of  $\alpha 1(I)$  procollagen synthesized by yeast is what would be expected for an unhydroxylated procollagen (R. A. Berg and D. J. Prockop, *Biochem. Biophys. Res. Commun.* (1973) 52:115-120).

An experiment to determine the Tm of SVWI-38-derived procollagen showed that 25 the triple helix was stable to greater than 37°C. Our earlier results showed that all of the yeast-produced human procollagen is secreted from the yeast cell and is located in the periplasm or media. Preprocollagen containing its native secretion signal sequence results in the most procollagen released from the periplasm.

#### Example 8

##### (Plasmid Copy Number Increase)

30 The expression vector system was constructed with a unique *NotI* site (located at coordinate 740 on the Gp5020 map; see Figure 1) to facilitate the addition of the *LEU2(d)* allele (J. D. Beggs, *Nature* (1978) 275:104-109; E. Erhart and C. P. Hollenberg, *J.*

*Bacteriology* (1983) 156: 625-635) or other elements that might benefit gene expression. We have also previously constructed a plasmid (Gp5091; see Figure 11) that contains the *LEU2(d)* allele as a *NotI* fragment (1369 bp). We have inserted the *LEU2(d)* allele in the various  $\alpha$ (I) procollagen expression plasmids.

5

### Example 9

#### ( $\alpha$ 2(I) Procollagen Yeast Expression Vector)

The human  $\alpha$ 2(I) procollagen cDNA is described in S.-T. Lee, B. D. Smith, D.S. Greenspan, *J. Biol. Chem.* (1988) 263:13414-13418. Plasmid Gp5219 contains this human  $\alpha$  2(I) procollagen cDNA operatively linked to the GAL 1-10 promoter in a plasmid which also 10 contains the PGK terminator, ampicillin gene and bacterial ori, and yeast 2  $\mu$  ori and yeast selectable marker TRP1 (see Figure 12). A new plasmid, Gp5220, was constructed containing the human  $\alpha$  1(I) procollagen cDNA and human  $\alpha$  2(I) procollagen cDNA operatively linked the GAL 1-10 promoter which also contains the PGK terminator, 15 ampicillin gene and bacterial ori, and yeast 2  $\mu$  ori and yeast selectable marker TRP1 (see Figure 12). These plasmids were transfected into yeast strains GY5196 and GY5198 and selected for transformants as described in Example 1. Selected transformants were grown, processed, and western blotted as described in Example 3. Results of the analysis showed the presence of antibody-reactive protein migrating in the expected location for  $\alpha$  2(I) 20 procollagen in the transformants from both yeast strains which contained the plasmids Gp5219 and Gp5220 using the antibody LF-116 (L. W. Fisher, *ibid.*). The presence of antibody-reactive protein migrating in the expected location for  $\alpha$  1(I) procollagen was observed only in the transformants in both yeast strains which contained the plasmid 25 Gp5220. The results show the expression of the human  $\alpha$  2(I) procollagen in transformants of both strains of yeast which harbor the  $\alpha$  2(I) procollagen cDNA, and also show the co-expression of the  $\alpha$  1(I) and  $\alpha$  2(I) procollagens in transformants of both strains of yeast 30 which harbor the  $\alpha$  1(I) and  $\alpha$  2(I) procollagen cDNAs. To determine whether the human  $\alpha$  1(I) and  $\alpha$  2(I) procollagen polypeptides synthesized in yeast are associating into the correct triple helical structure, yeast extracts containing plasmid Gp5220 were digested with pepsin as described in Example 7. The results show the triple helical region was resistant to pepsin at 20 and 25°C. Bands corresponding to the expected size of  $\alpha$  1(I) and  $\alpha$  2(I) collagen were observed, which indicates the triple helix was composed of both  $\alpha$  1(I) and  $\alpha$  2(I) procollagen polypeptides.

Example 10

## (Prolyl Hydroxylase Yeast Expression Vector)

The chicken prolyl hydroxylase cDNA is described in J. A. Bassuk, W. W.-Y. Kao, P. Herzer, N. L. Kedersha, J. Sayer, J. A. DeMartino, B. L. Daugherty, G.E. Mark III, R. A. Berg, Proc. Nat. Acad. Sci. (1989) 86:7382-7386. Plasmid Gp5217 contains this chicken prolyl hydroxylase cDNA operatively linked to the GAL 1-10 promoter in a plasmid which also contains the PGK terminator, ampicillin gene and bacterial ori, and yeast 2  $\mu$  ori and yeast selectable marker TRP1 (see Figure 13). A new plasmid, Gp5218 contains this chicken prolylhydroxylase and human  $\alpha$ 1(I) procollagen cDNAs operatively linked the GAL 1-10 promoter which also contains the PGK terminator, ampicillin gene and bacterial ori, and yeast 2  $\mu$  ori and yeast selectable marker TRP1 (see Figure 13). These plasmids were transfected into yeast strains GY5196 and GY5198 and selected for transformants as described in Example 1. Selected transformants were grown, processed, and western blotted as described in Example 3. Results of the analysis showed the presence of antibody-reactive protein migrating in the expected location for chicken prolyl hydroxylase in the transformants from both yeast strains which contained the plasmids Gp5217 and Gp5218 using an antibody produced in immunized rabbits against the purified chicken prolyl hydroxylase. The presence of antibody-reactive protein migrating in the expected location for  $\alpha$  1(I) procollagen was observed only in the transformants in both yeast strains which contained the plasmid Gp5218. The results show the expression of the chicken prolyl hydroxylase in both strains of yeast which harbor the chicken prolyl hydroxylase cDNA, and also show the co-expression of the  $\alpha$  1(I) procollagen and prolyl hydroxylase in transformants of both strains of yeast which harbor the  $\alpha$  1(I) procollagen and prolyl hydroxylase cDNAs. Furthermore, yeast containing the chicken prolyl hydroxylase cDNA were fractionated into intracellular, periplasmic, and media fractions as described in Example 6. Virtually all the prolyl hydroxylase was found in the intracellular fraction.

Example 11

## (Protein Disulfide Isomerase Yeast Expression Vector)

The chicken protein disulfide isomerase (PDI) cDNA is described in W. W.-T. Kao, M. Nakazawa, T. Aida, W. V. Everson, C. W.-C. Kao, J. M. Seyer, S. H. Hughes, Conn. Tissue. Res. (1988) 18:157-174. Plasmid Gp5349 contains this chicken PDI cDNA with a preinvertase signal sequence operatively linked to the PGK promoter and ADH terminator,

chicken prolyl hydroxylase and human  $\alpha$  1(I) procollagen cDNAs operatively linked to the GAL 1-10 promoter, a PGK-terminator operatively linked to the prolyl hydroxylase cDNA which also contains the ampicillin gene and bacterial ori, and yeast 2  $\mu$  ori and yeast selectable marker TRP1 (see Figure 14). These plasmids were transfected into yeast strains 5 GY5196 and GY5198 and selected for transformants as described in Example 1. Selected transformants were grown, processed, and western blotted as described in Example 3. Results of the analysis showed the presence of antibody-reactive protein migrating in the expected location for chicken PDI using an antibody produced in immunized rabbits against the purified chicken PDI, in the expected location for chicken prolyl hydroxylase using 10 antibodies described previously, and in the expected location for human  $\alpha$  1(I) procollagen using antibodies described in Example 10. To determine whether the human  $\alpha$  1(I) procollagen polypeptides synthesized in yeast are associating into the correct triple helical structure, yeast extracts containing plasmid Gp5349 were digested with pepsin as described in Example 7. The results show the triple helical region was resistant to up to and including 15 35°C. Bands corresponding to the expected size of  $\alpha$  1(I)collagen were observed, which provides indirect evidence the triple helix, composed of  $\alpha$  1(I) procollagen polypeptides, was hydroxylated at proline residues which increases the thermal stability of the collagen triple helix. Direct evidence of hydroxyproline residues in the procollagen polypeptide was obtained through purification and amino acid composition analysis (REF) of the procollagen. 20

All publications and patent applications cited in this specification are herein incorporated by reference, as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in 25 the art, in light of the teachings of this invention, that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. A method for making a mammalian collagen or procollagen in yeast, said method comprising the steps of:

5 (a) incubating a recombinant yeast cell comprising (i) a stable genetic construct comprising a mammalian collagen gene comprising a secretion signal sequence and a promoter heterologous to said collagen gene, said collagen gene operably linked to said promoter, in a medium under conditions wherein said collagen gene is expressed as a collagen or procollagen chain;

(b) recovering said collagen or procollagen chain.

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2. The method of claim 1, wherein in said incubating step said chain is assembled into triple helical collagen or procollagen and wherein said recovering step comprises recovering said chain in the form of said triple helical collagen or procollagen.

15

3. The method of claim 1, wherein in said incubating step said chain is secreted through the plasma membrane and released from the cell wall of said yeast into the medium in the form of triple helical collagen or procollagen and wherein said recovering step comprises recovering said chain in the form of said triple helical collagen or procollagen.

20

4. The method of claim 1, wherein said secretion signal sequence is heterologous to said collagen gene.

5. The method of claim 1, wherein said incubating step is performed at between about 28 °C and about 37 °C.

25

6. The method of claim 1, wherein said medium comprises a supplemental nutrient sufficient to promote the growth of said yeast cell under said conditions.

30

7. The method of claim 1, wherein said medium comprises a supplemental nutrient sufficient to promote the growth of said yeast cell under said conditions, said nutrient selected from the group consisting of an amino acid, a carbon source, a nitrogen source, a vitamin, a cofactor or a mineral.

8. The method of claim 1, wherein said construct further comprises a selectable marker.
9. The method of claim 1, wherein said construct further comprises a selectable marker, wherein said selectable marker is selected from the group consisting of an invertase gene, 5 *LEU2(d)*, *TRP1*, *URA3*, *HIS3*, and combinations thereof.
10. The method of claim 1, wherein said yeast cell is a *Saccharomyces cerevisiae*.
11. The method of claim 1, wherein said yeast cell has at least one genetic mutation that 10 causes the cell wall of said yeast cell to exhibit increased permeability to collagen and procollagen.
12. The method of claim 1, wherein said yeast cell has at least one genetic mutation that causes the cell wall of said yeast cell to exhibit increased permeability to collagen and 15 procollagen, wherein said genetic mutation effects the presence or function of a cell wall component selected from a chitin, a glucan and a cell wall glycoprotein.
13. The method of claim 3, wherein substantially all of said procollagen or collagen 20 chains expressed by said yeast cell are secreted through the plasma membrane and cell wall of said yeast cell.
14. The method of claim 1, wherein said yeast cell further comprises (ii) a prolyl hydroxylase gene and (iii) a heterologous protein disulfide isomerase gene, wherein said prolyl hydroxylase gene is stably expressed to produce a prolyl hydroxylase and said protein disulfide isomerase gene is stably expressed to produce a protein disulfide isomerase, and 25 said hydroxylase and said isomerase associate in an active complex in the endoplasmic reticulum of said cell, and said complex hydroxylates prolyl residues of said collagen or procollagen chain.
- 30 15. The method of claim 1, wherein said mammalian collagen gene comprises a natural human collagen gene sequence.

16. A method for making a human collagen or procollagen in yeast, said method comprising the steps of:

(a) incubating a recombinant yeast cell comprising (i) a stable genetic construct comprising a mammalian collagen gene comprising a secretion signal sequence heterologous to said collagen gene, a promoter heterologous to said collagen gene, and a selectable marker, said collagen gene being operably linked to said promoter, (ii) a prolyl hydroxylase gene, and (iii) a protein disulfide isomerase gene in a medium under conditions wherein said prolyl hydroxylase gene is stably expressed to produce a prolyl hydroxylase and said protein disulfide isomerase gene is stably expressed to produce a protein disulfide isomerase, and 5 said hydroxylase and said isomerase associate in an active complex in the endoplasmic reticulum of said cell; wherein said yeast cell is *Saccharomyces cerevisiae*; wherein said collagen gene is expressed as a procollagen or collagen chain and said complex hydroxylates prolyl residues of said collagen or procollagen chain; wherein substantially all of said procollagen or collagen chains expressed by said yeast cell are secreted through the plasma 10 membrane and released from the cell wall of said yeast cell into the medium in the form of a triple helical procollagen or collagen; and

15 (b) recovering said triple helical collagen or procollagen.

17. A recombinant yeast cell useful for making a collagen or procollagen protein comprising: (i) a stable genetic construct comprising a mammalian collagen gene comprising a secretion signal sequence, a promoter heterologous to said collagen gene, wherein said collagen gene is operably linked to said promoter.

18. The recombinant yeast cell of claim 17, wherein said secretion signal sequence is 25 heterologous to said collagen gene.

19. The recombinant yeast cell of claim 17, wherein said construct further comprises a selectable marker.

30 20. The recombinant yeast cell of claim 17, wherein said construct further comprises a selectable marker, wherein said selectable marker is selected from the group consisting of an invertase gene, *LEU2(d)*, *TRP1*, *URA3*, *HIS3*, and combinations thereof.

21. The recombinant yeast cell of claim 17, wherein said yeast cell is a *Saccharomyces cerevisiae*.

22. The recombinant yeast cell of claim 17, wherein said yeast cell has at least one 5 genetic mutation that causes the cell wall of said yeast cell to exhibit increased permeability to collagen and procollagen.

23. The recombinant yeast cell of claim 17, wherein said yeast cell has at least one 10 genetic mutation that causes the cell wall of said yeast cell to exhibit increased permeability to collagen and procollagen, wherein said genetic mutation effects the presence or function of a cell wall component selected from chitin, glucan synthetase, and a cell wall glycoprotein.

24. A recombinant yeast cell according to claim 17 further comprising (ii) a prolyl 15 hydroxylase gene and (iii) a heterologous protein disulfide isomerase gene, wherein said prolyl hydroxylase gene is stably expressed to produce a prolyl hydroxylase and said protein disulfide isomerase gene is stably expressed to produce a protein disulfide isomerase, and said hydroxylase and said isomerase associate in an active complex in the endoplasmic reticulum of said cell, and said complex hydroxylates prolyl residues of said collagen or procollagen chain.

20  
25. A recombinant yeast cell according to claim 17, wherein said mammalian collagen gene comprises a human collagen gene sequence.

26. A recombinant yeast cell useful for making a human collagen or procollagen 25 comprising: (i) a stable genetic construct comprising a human collagen gene comprising a secretion signal sequence heterologous to said collagen gene, a promoter heterologous to said collagen gene, and a selectable marker, wherein said collagen gene is operably linked to said promoter, (ii) a prolyl hydroxylase gene, and (iii) a heterologous protein disulfide isomerase gene; wherein said yeast cell is *Saccharomyces cerevisiae*.

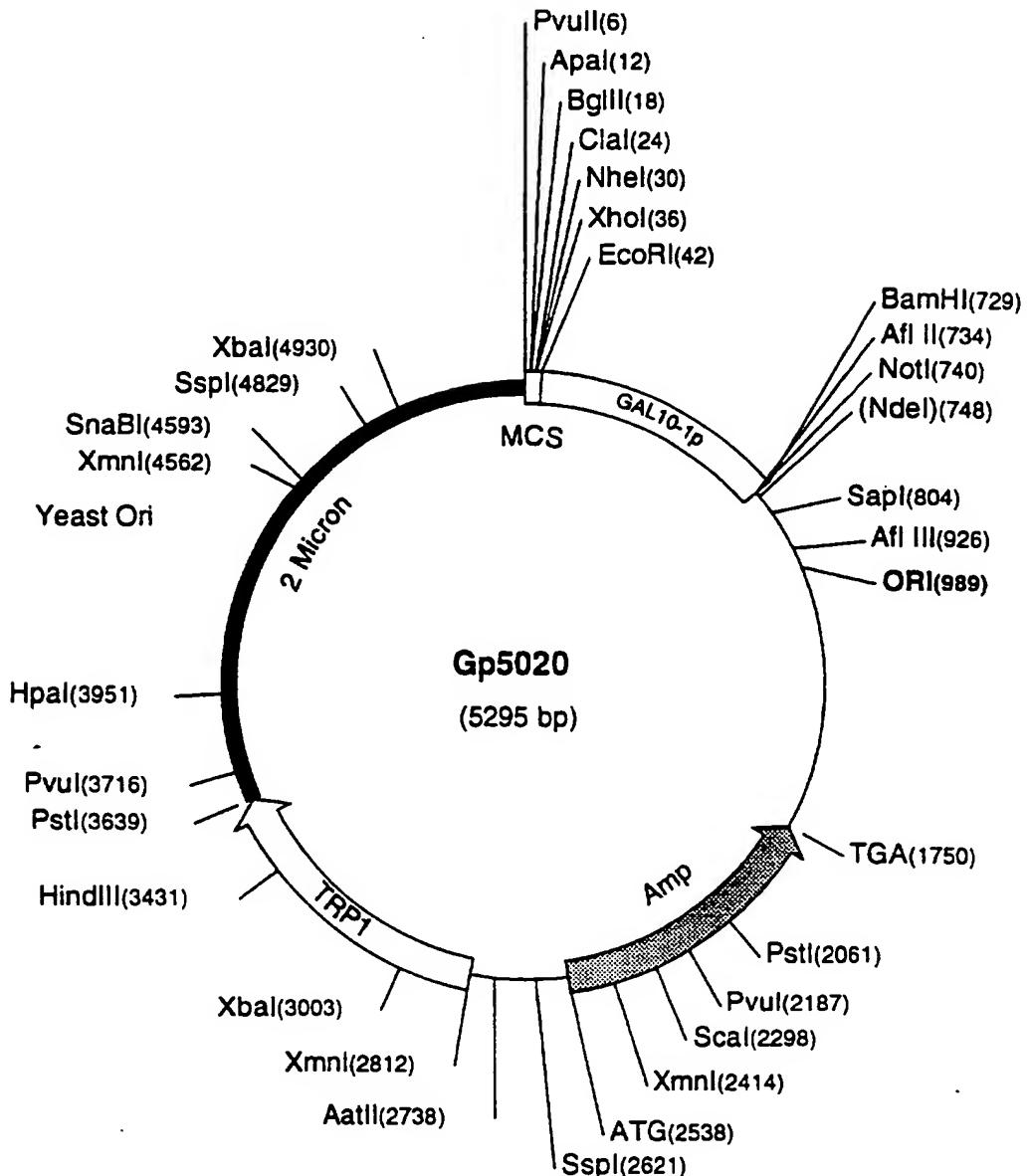


Figure 1

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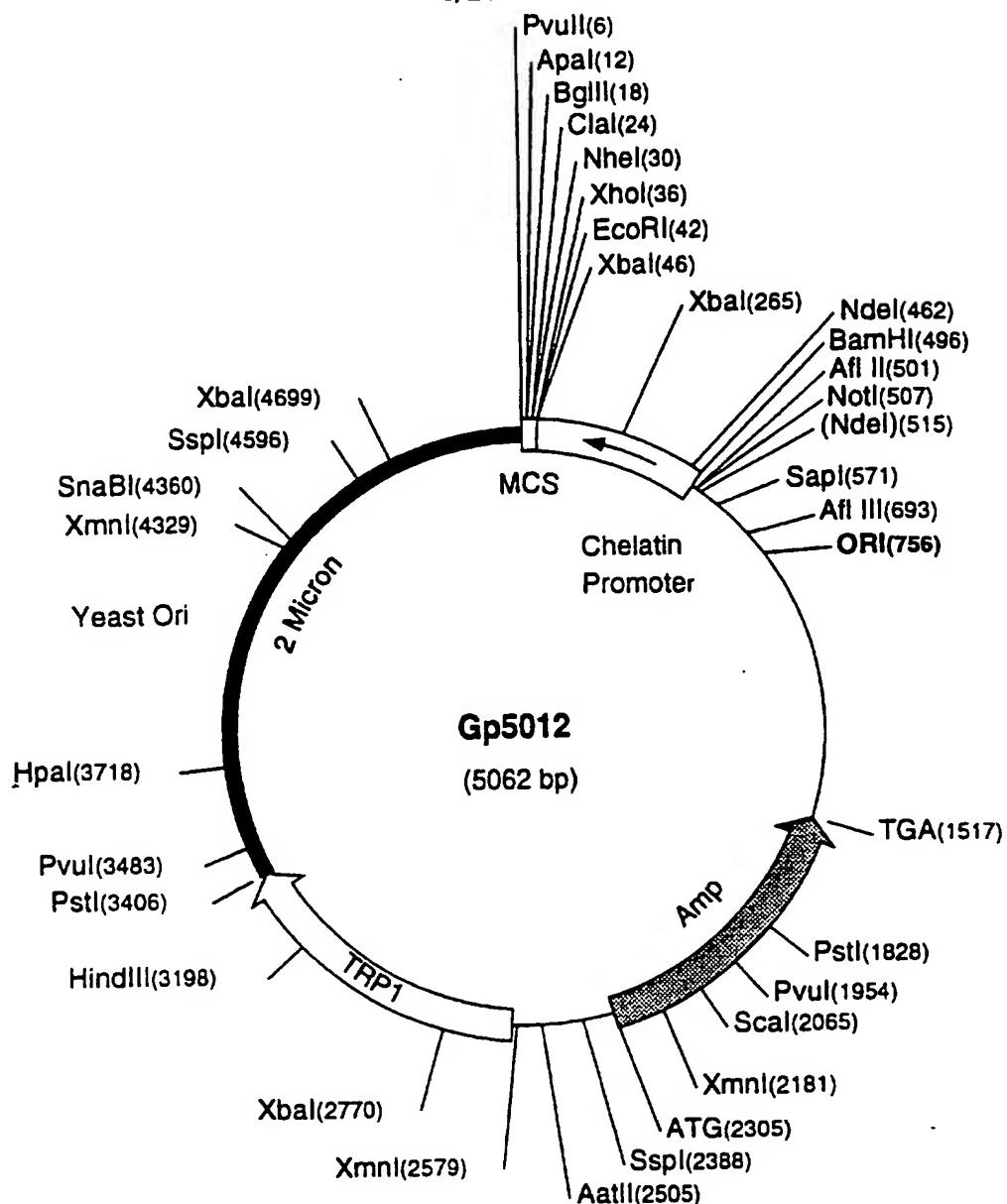


Figure 2

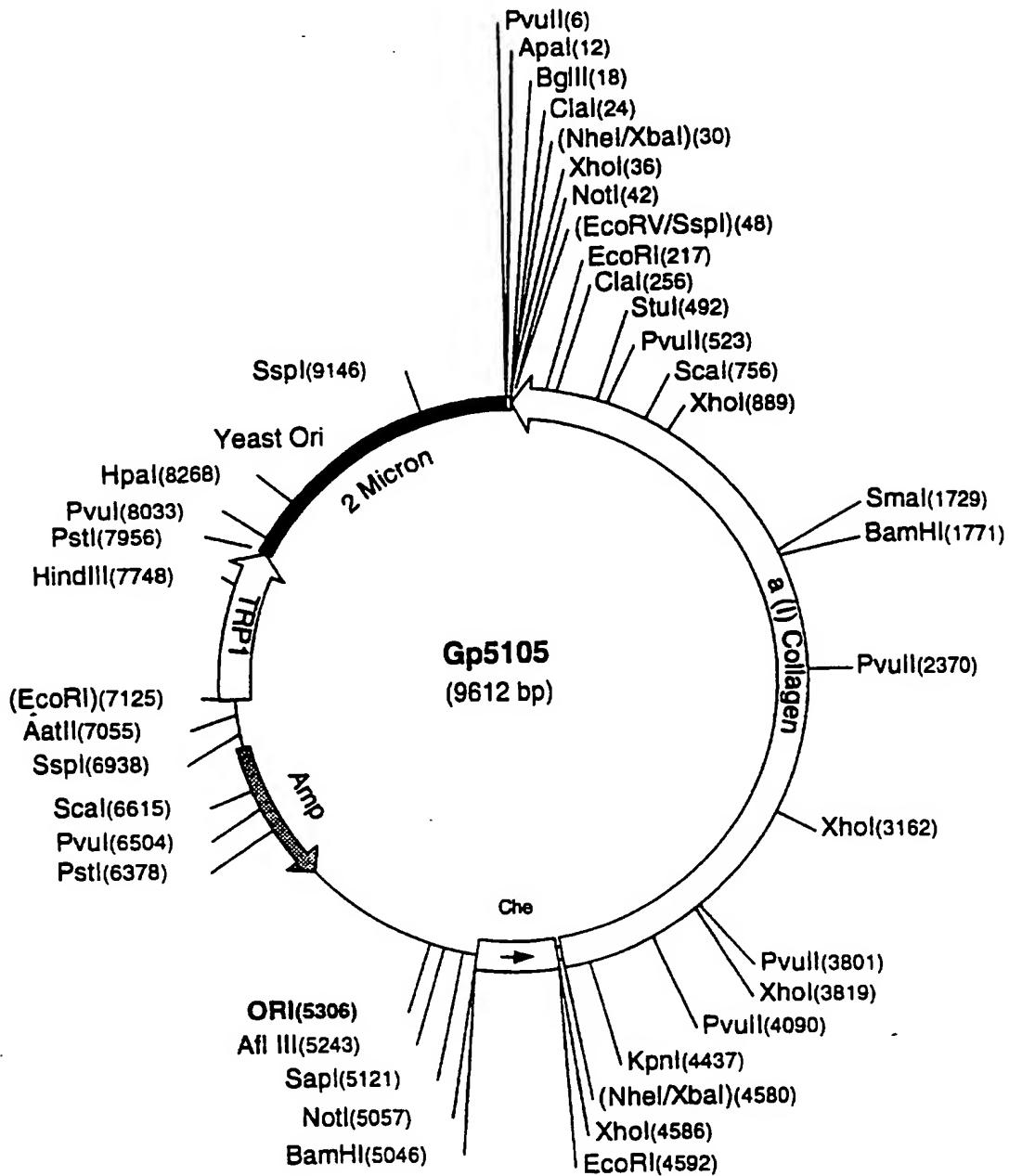


Figure 3.

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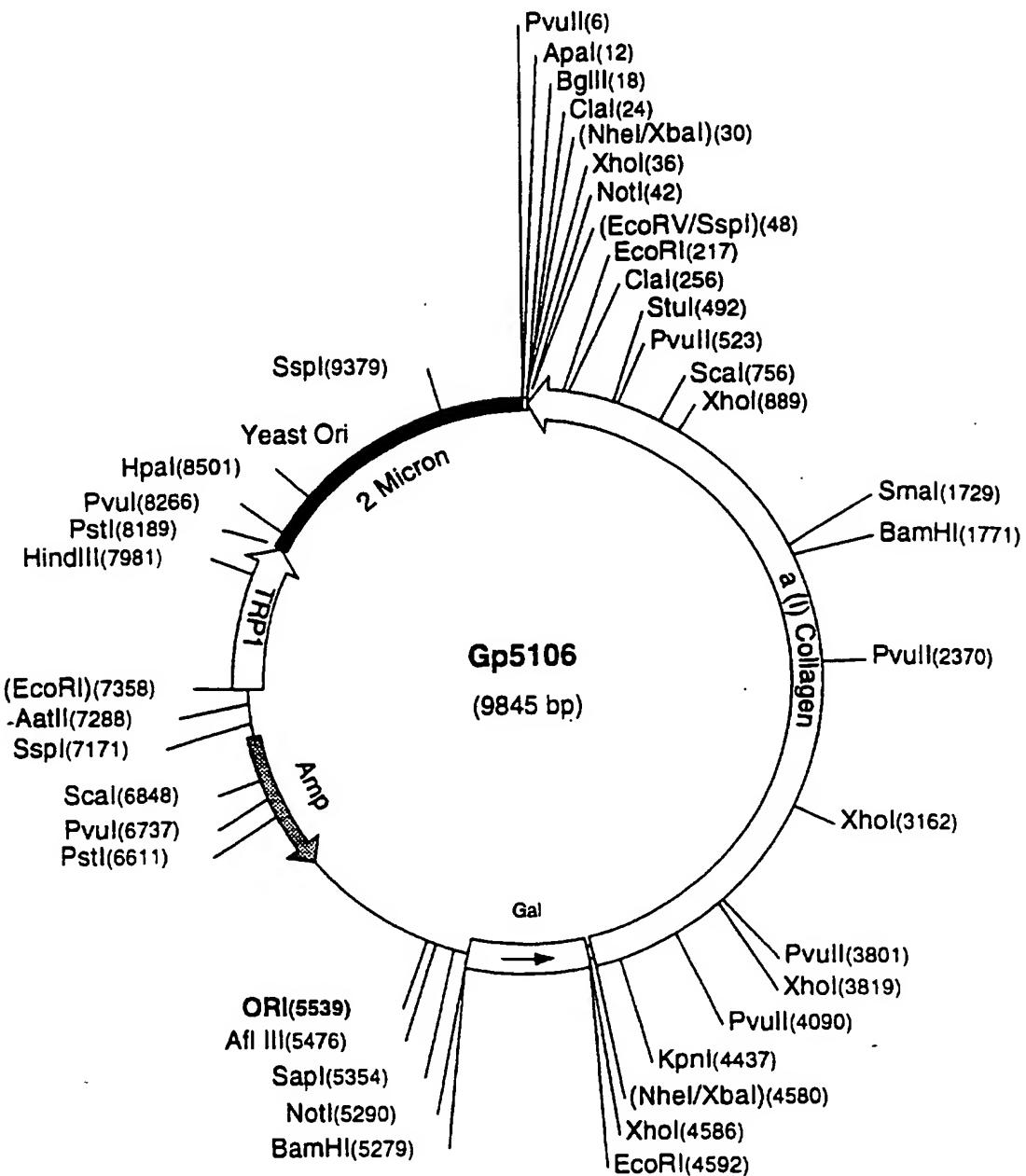


Figure 4

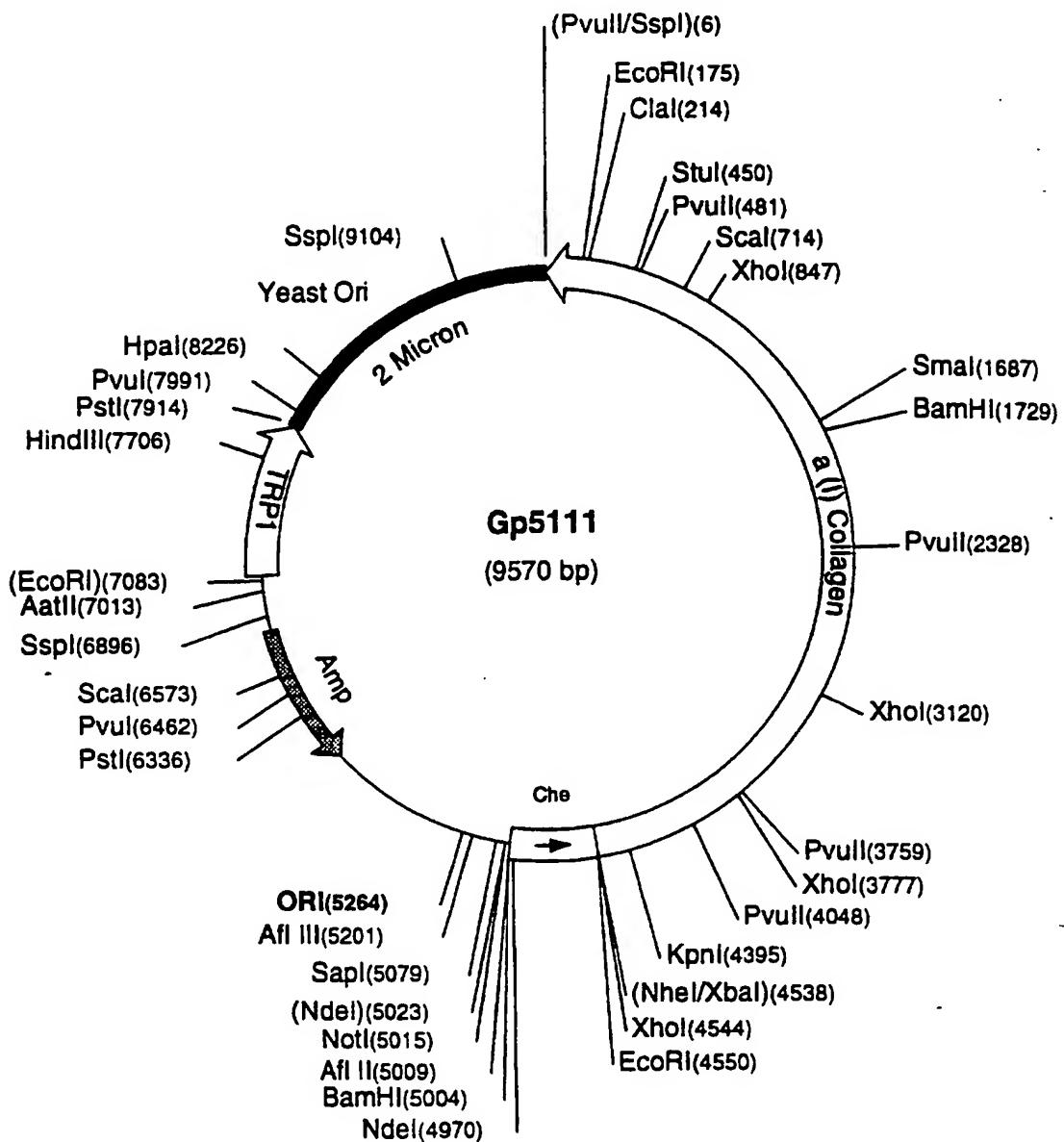


Figure 5.

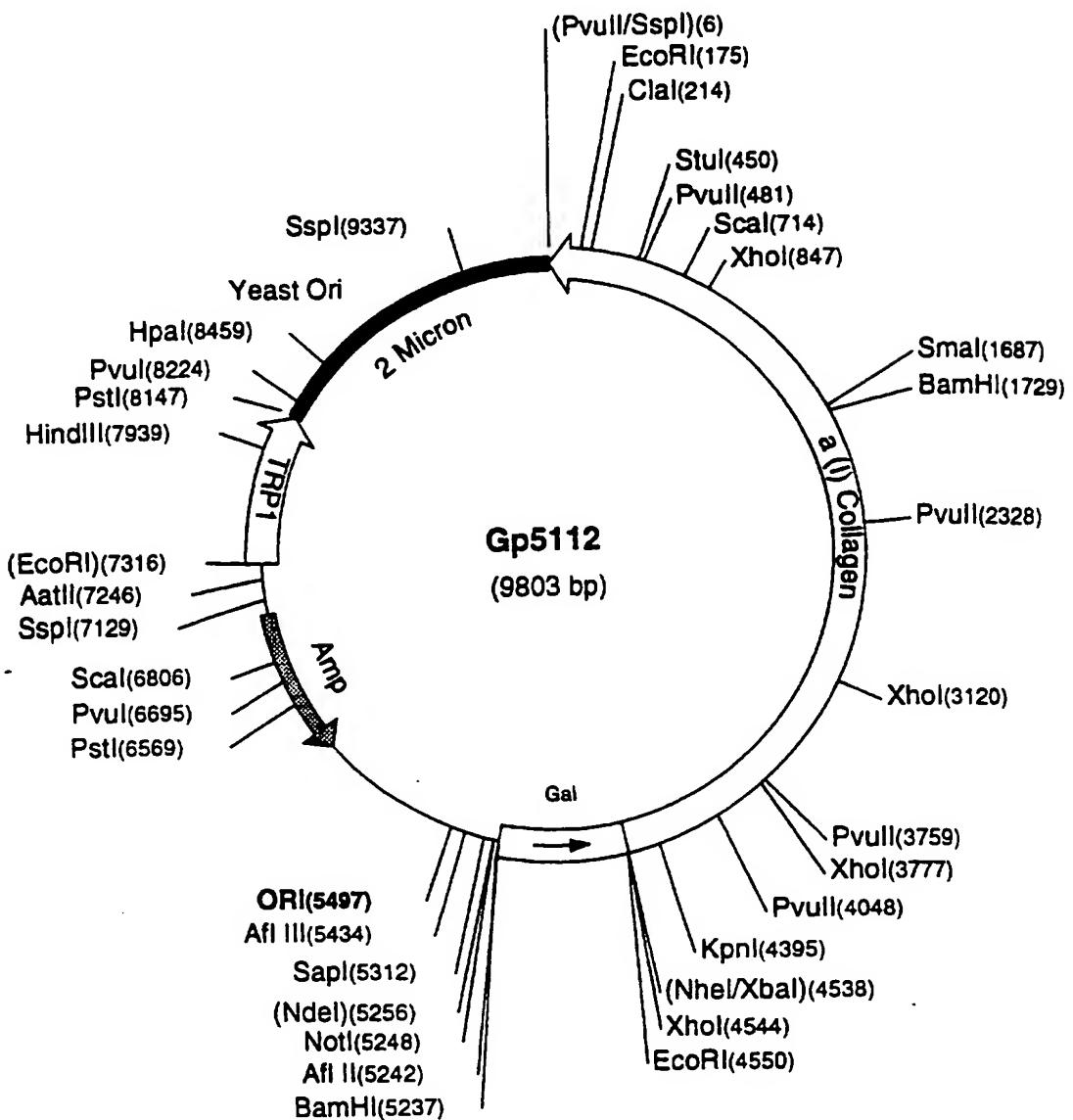


Figure 6

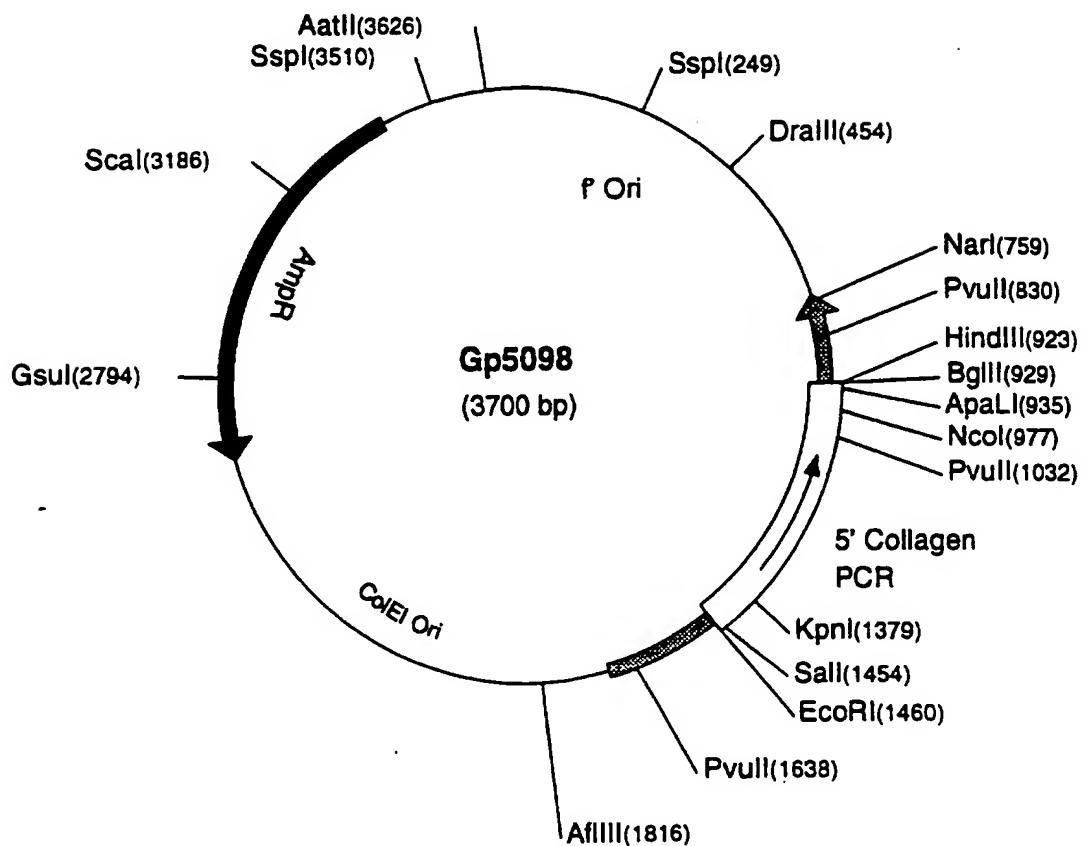


Figure 7.

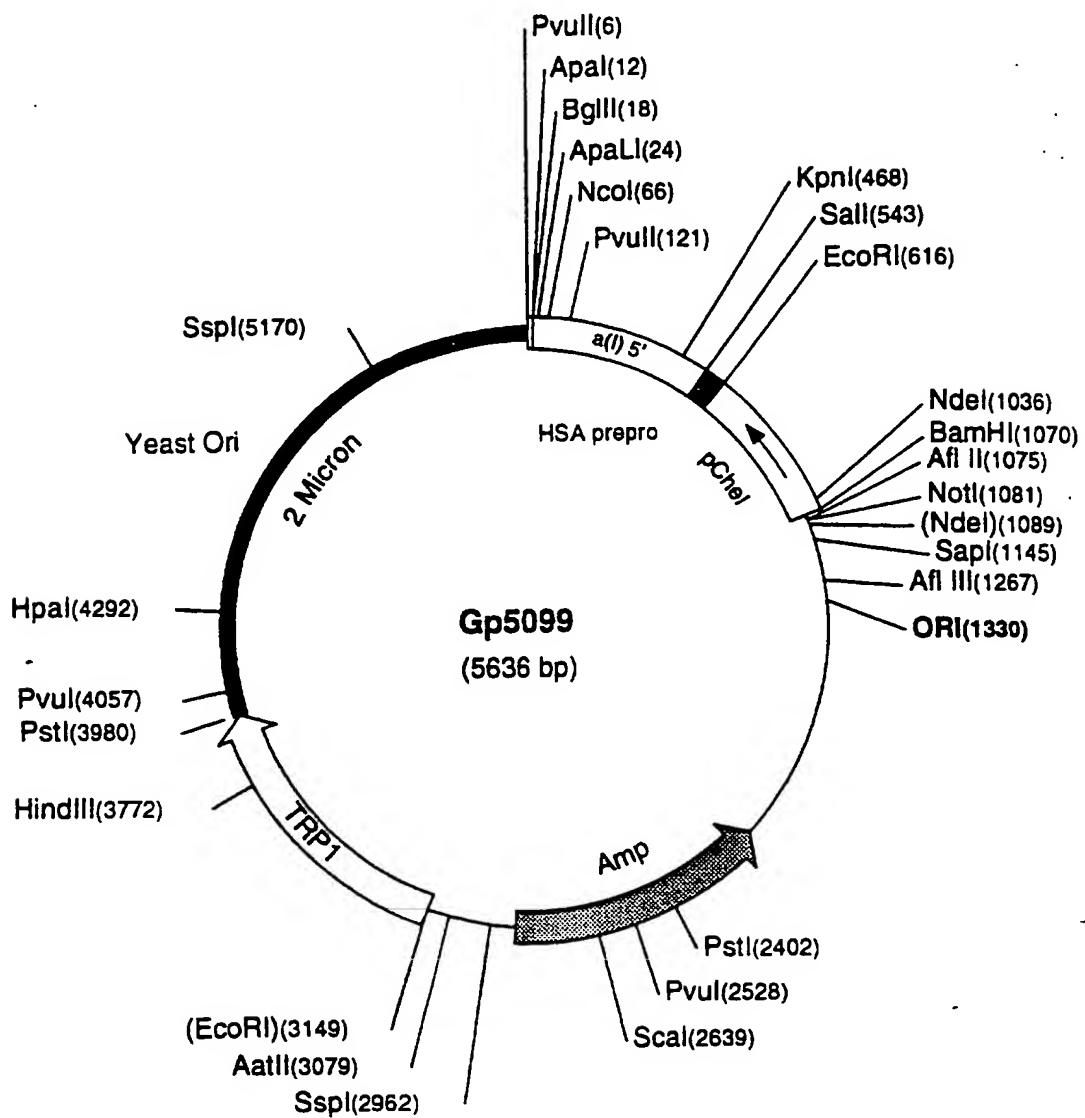


Figure 8

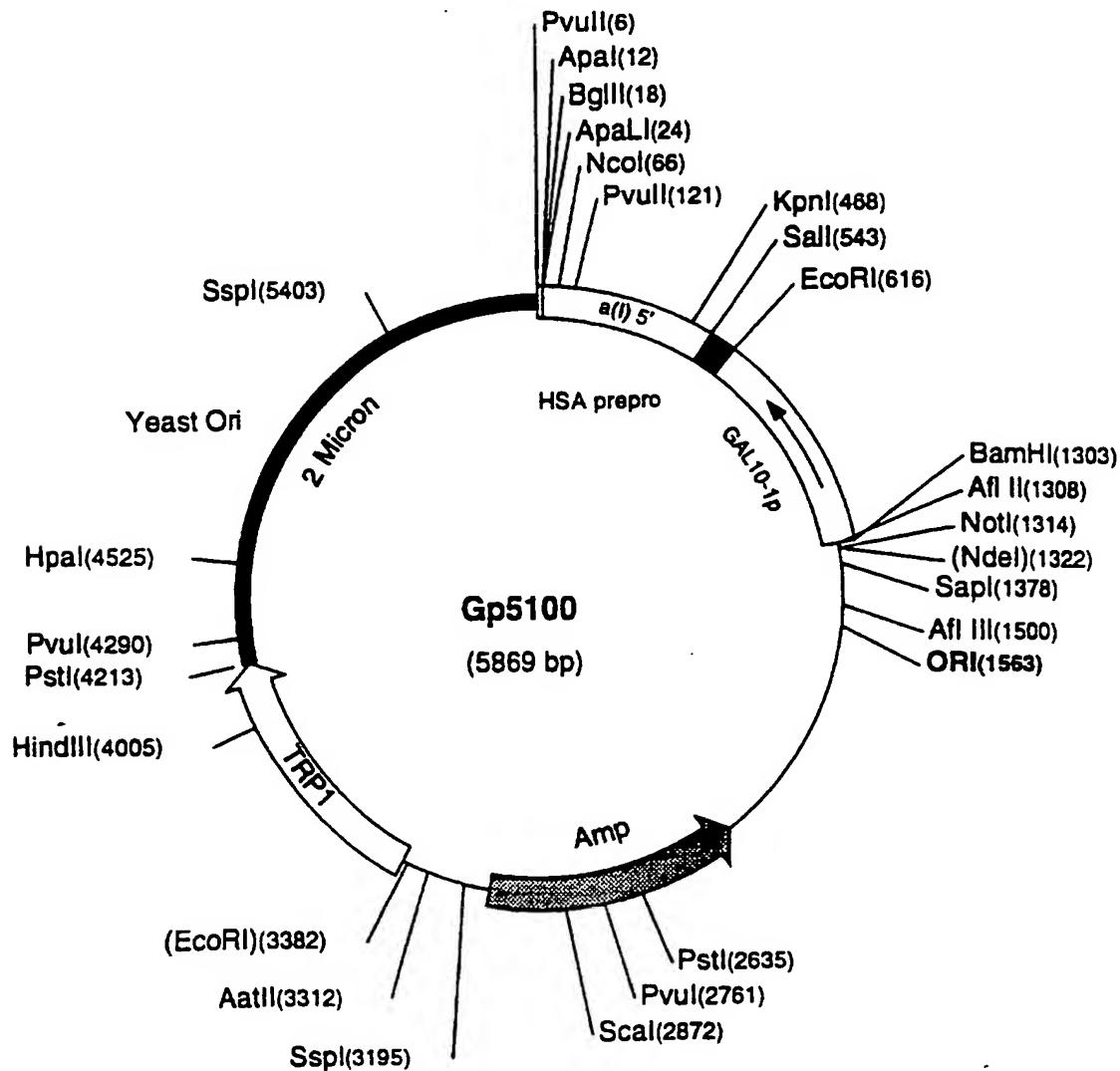


Figure 9.

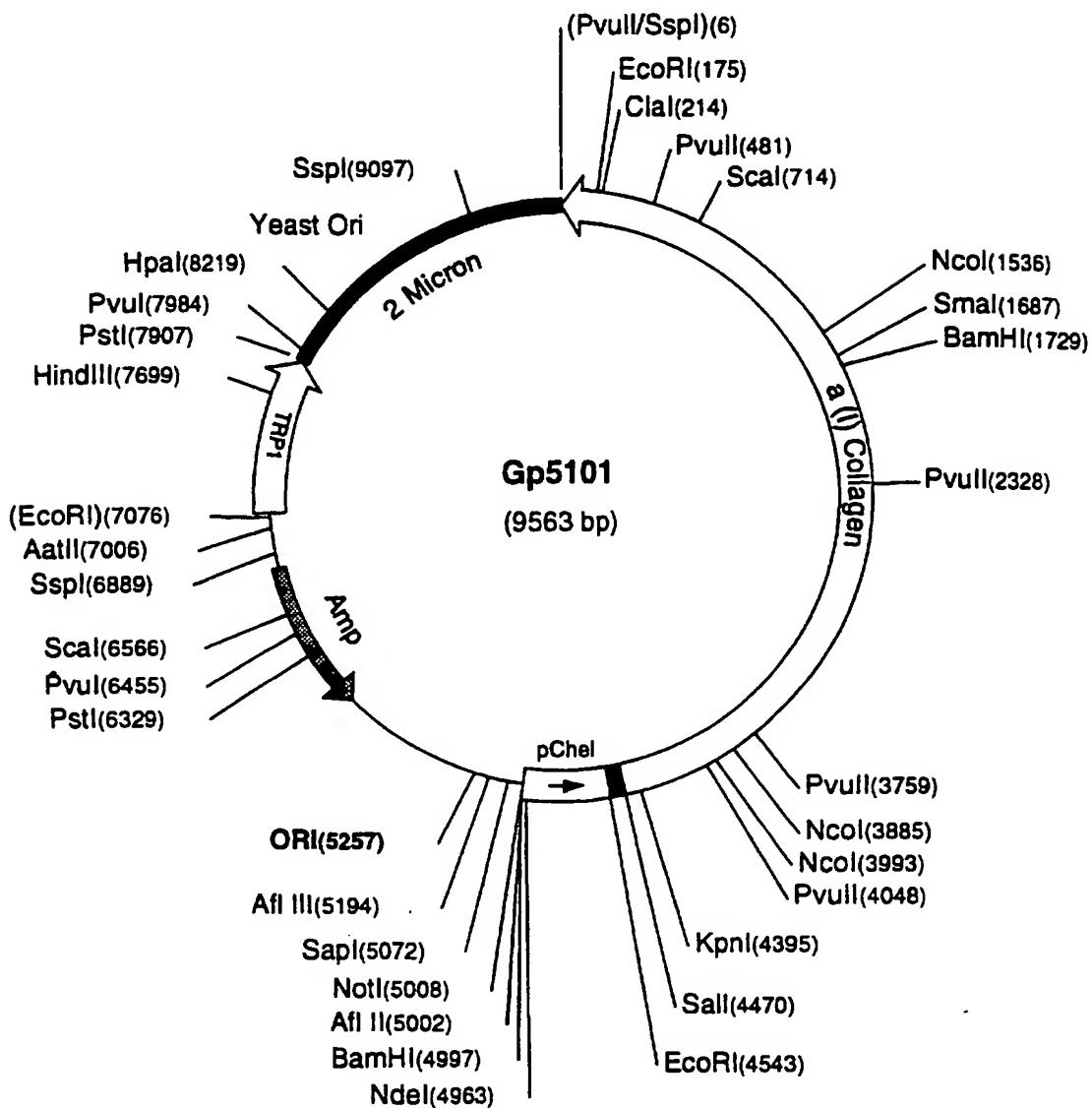


Figure 10

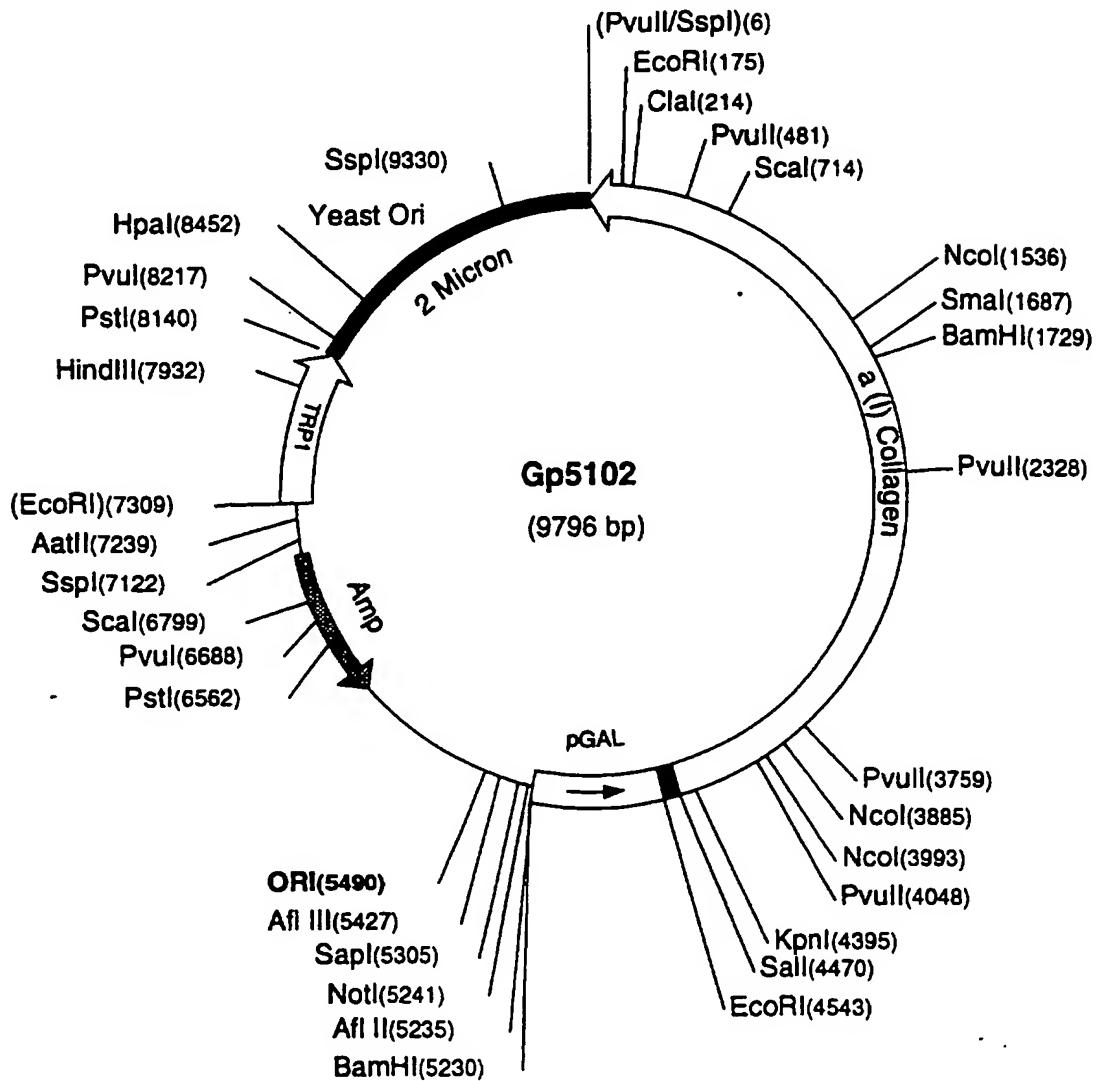


Figure 11

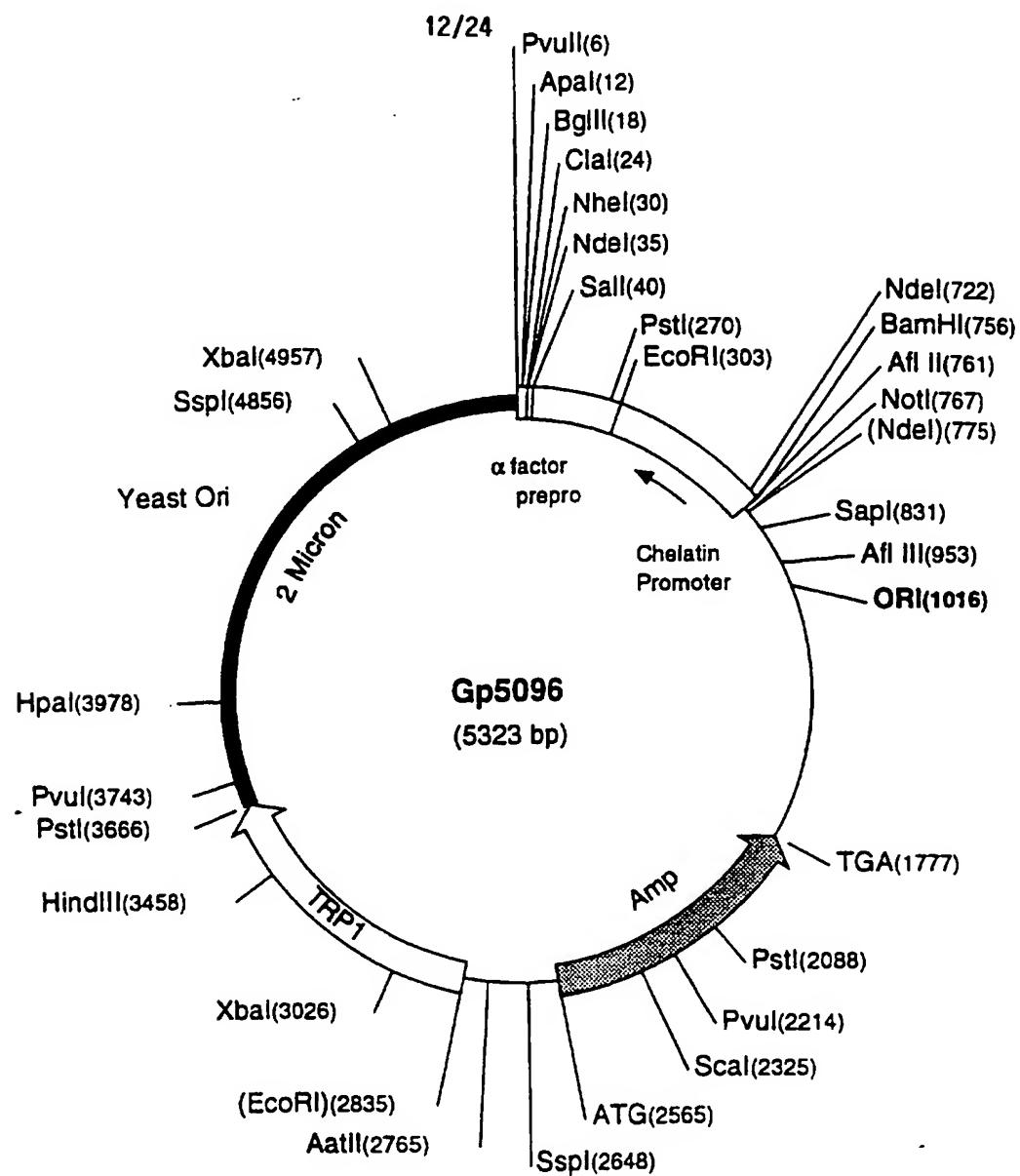


Figure 12

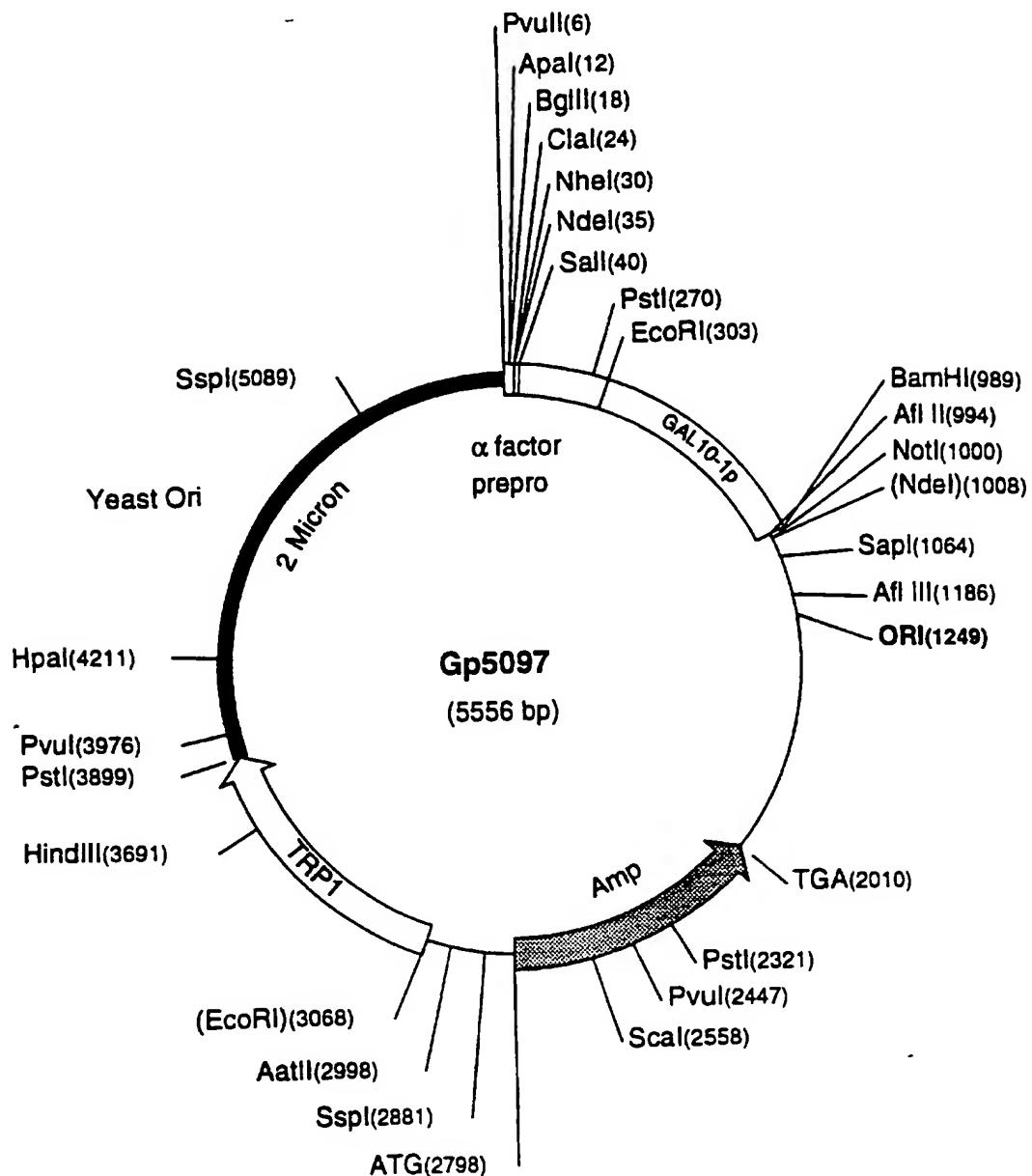


Figure 13

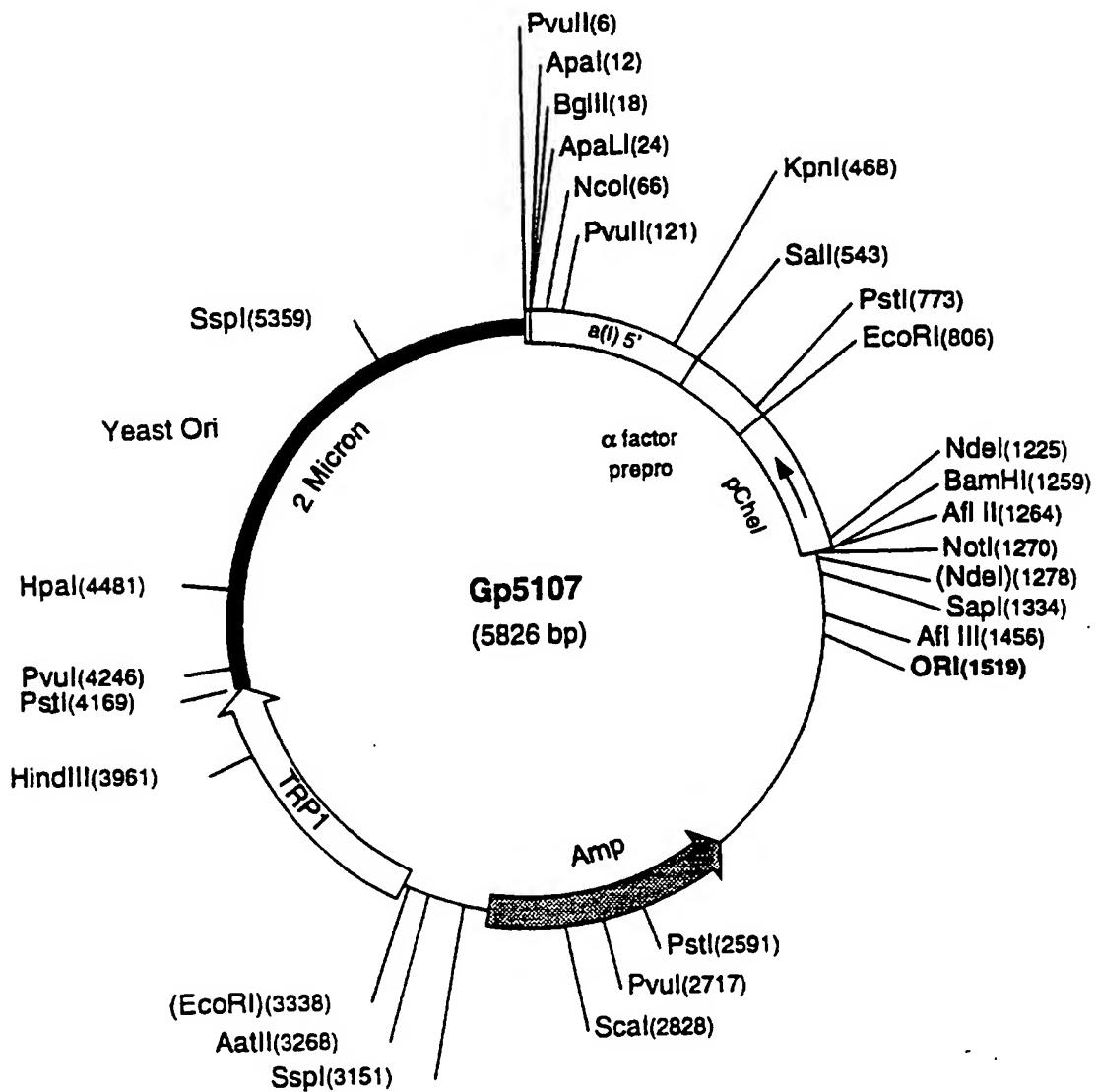


Figure 14

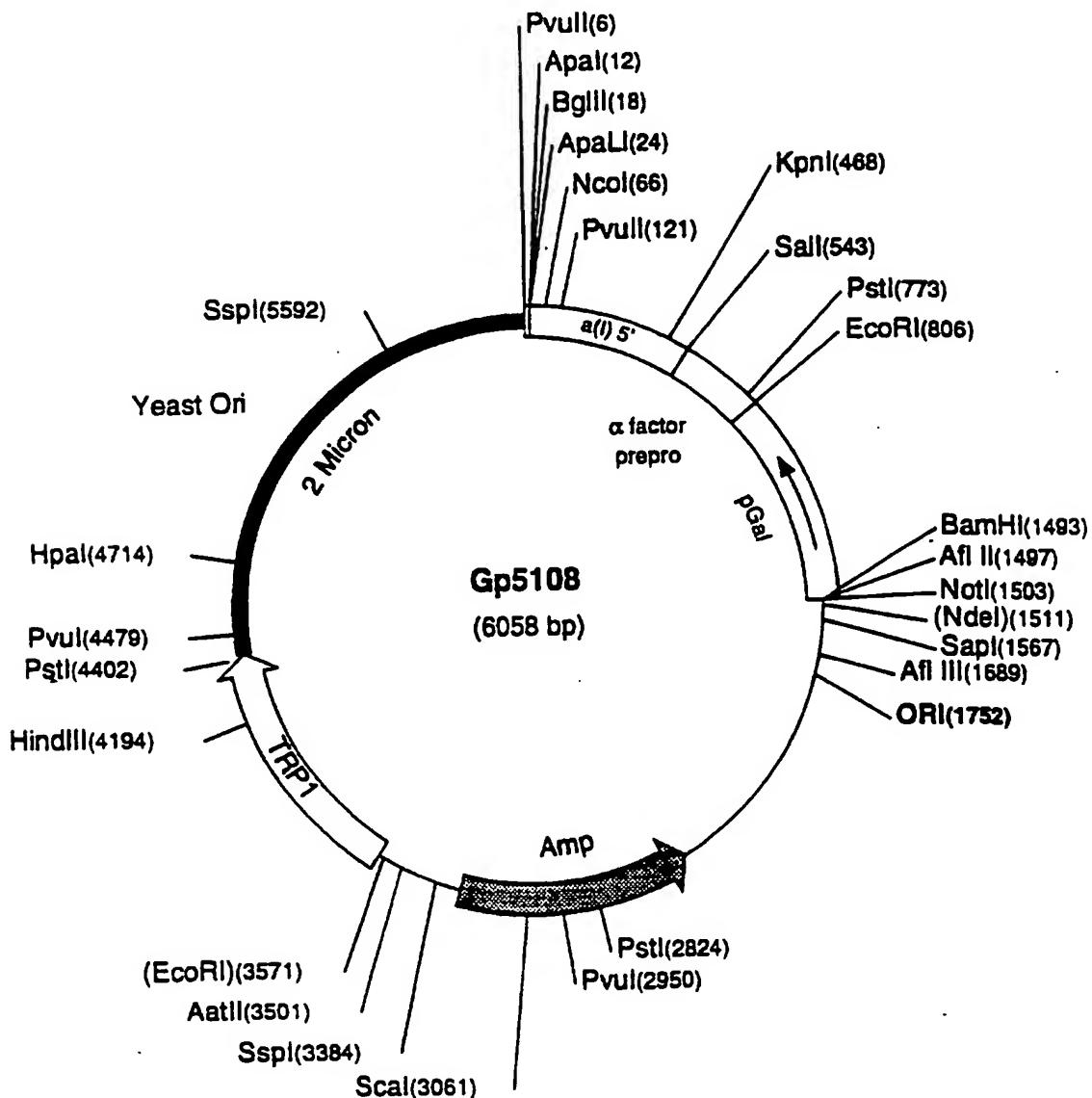


Figure 15

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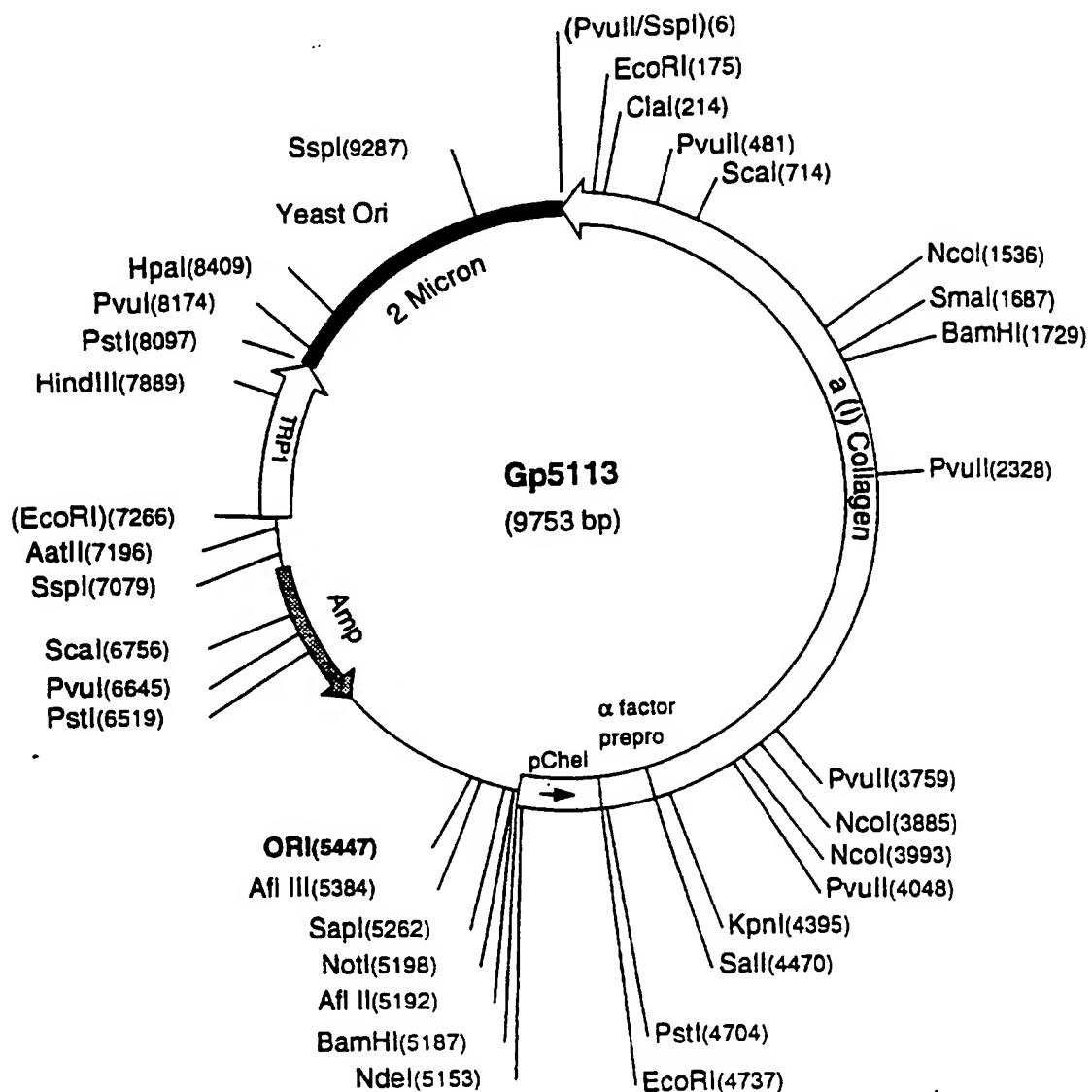


Figure 16

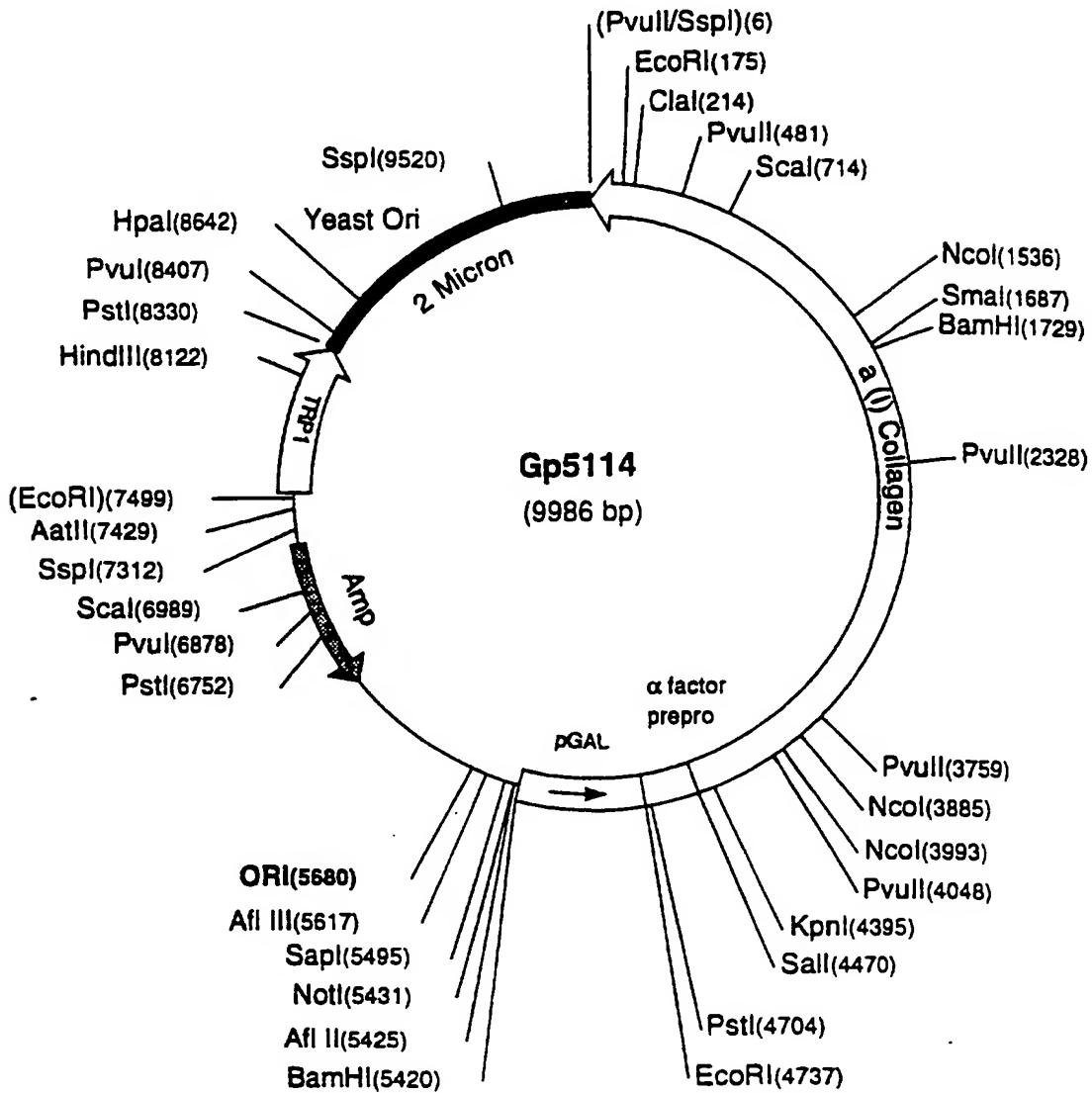


Figure 17

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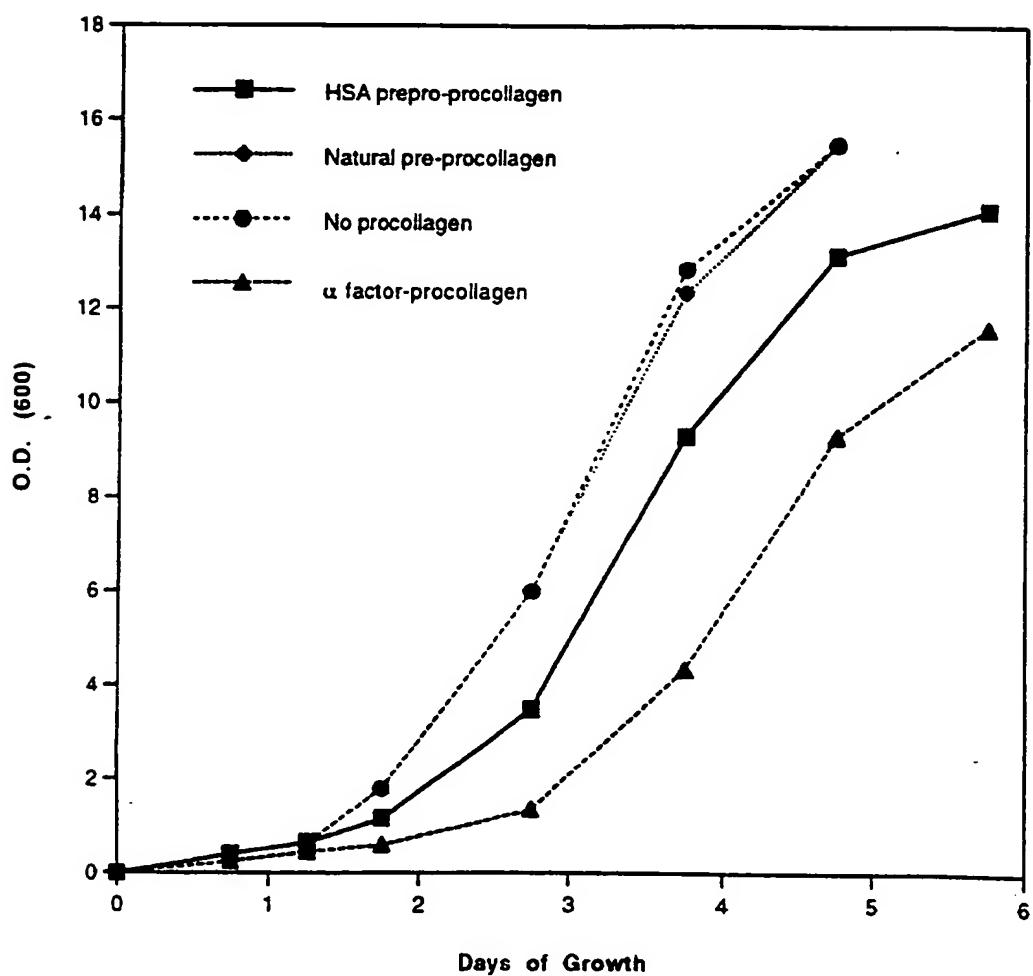


Figure 18/

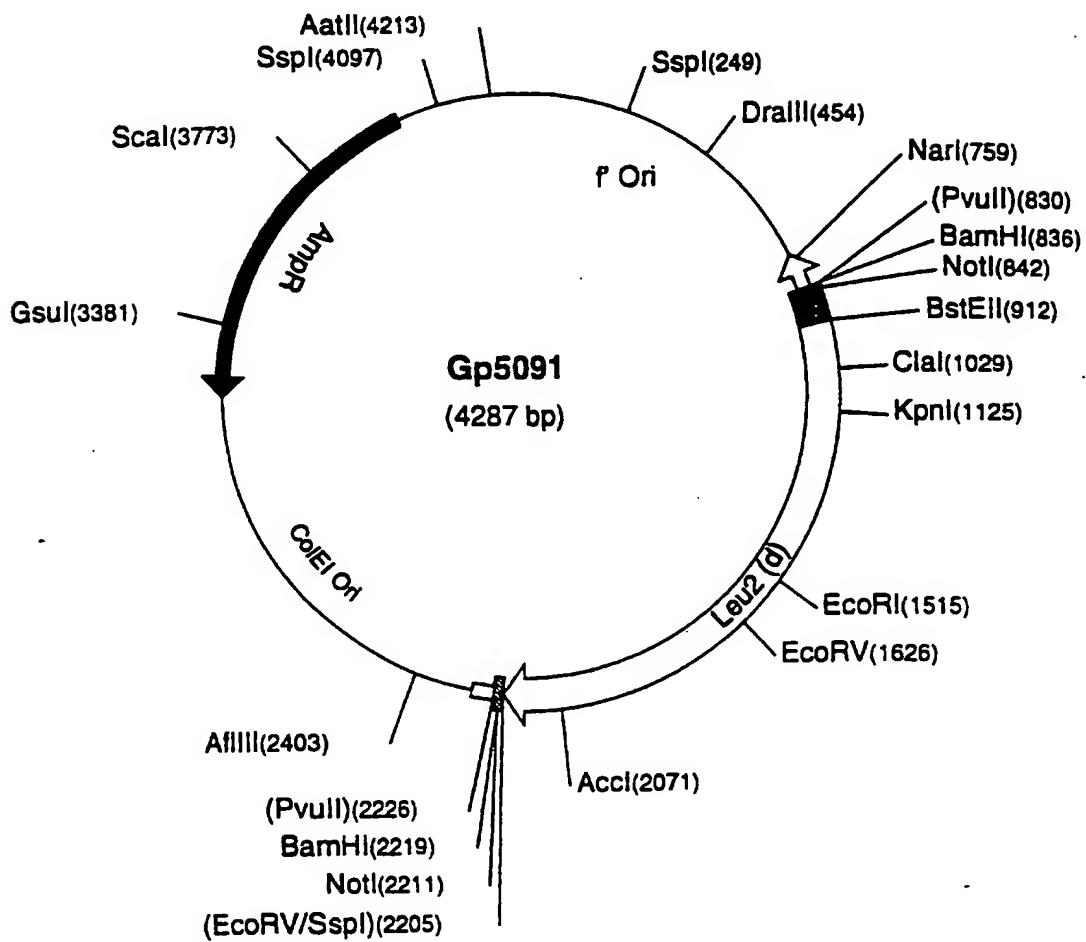


Figure 19

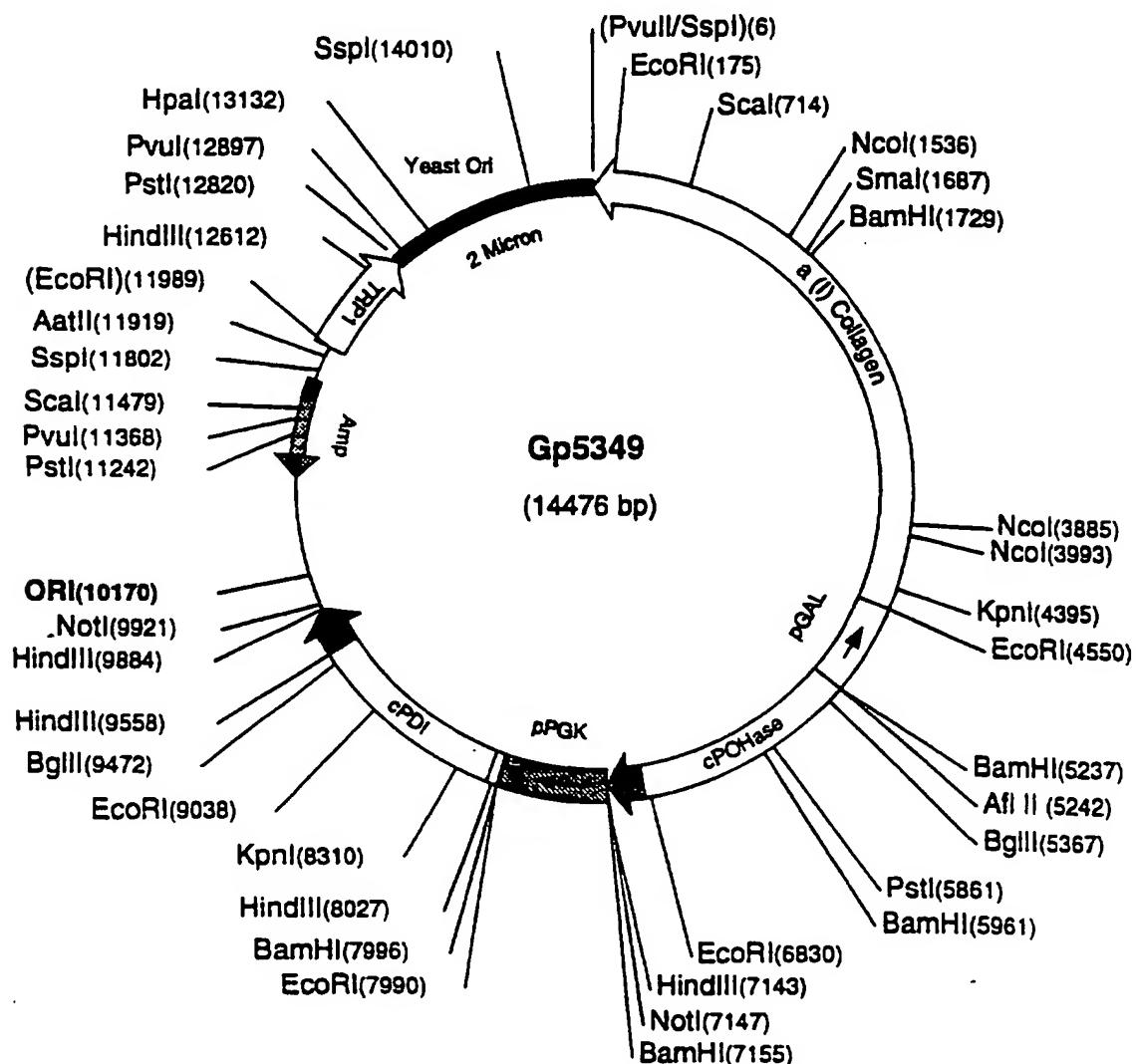


Figure 20,

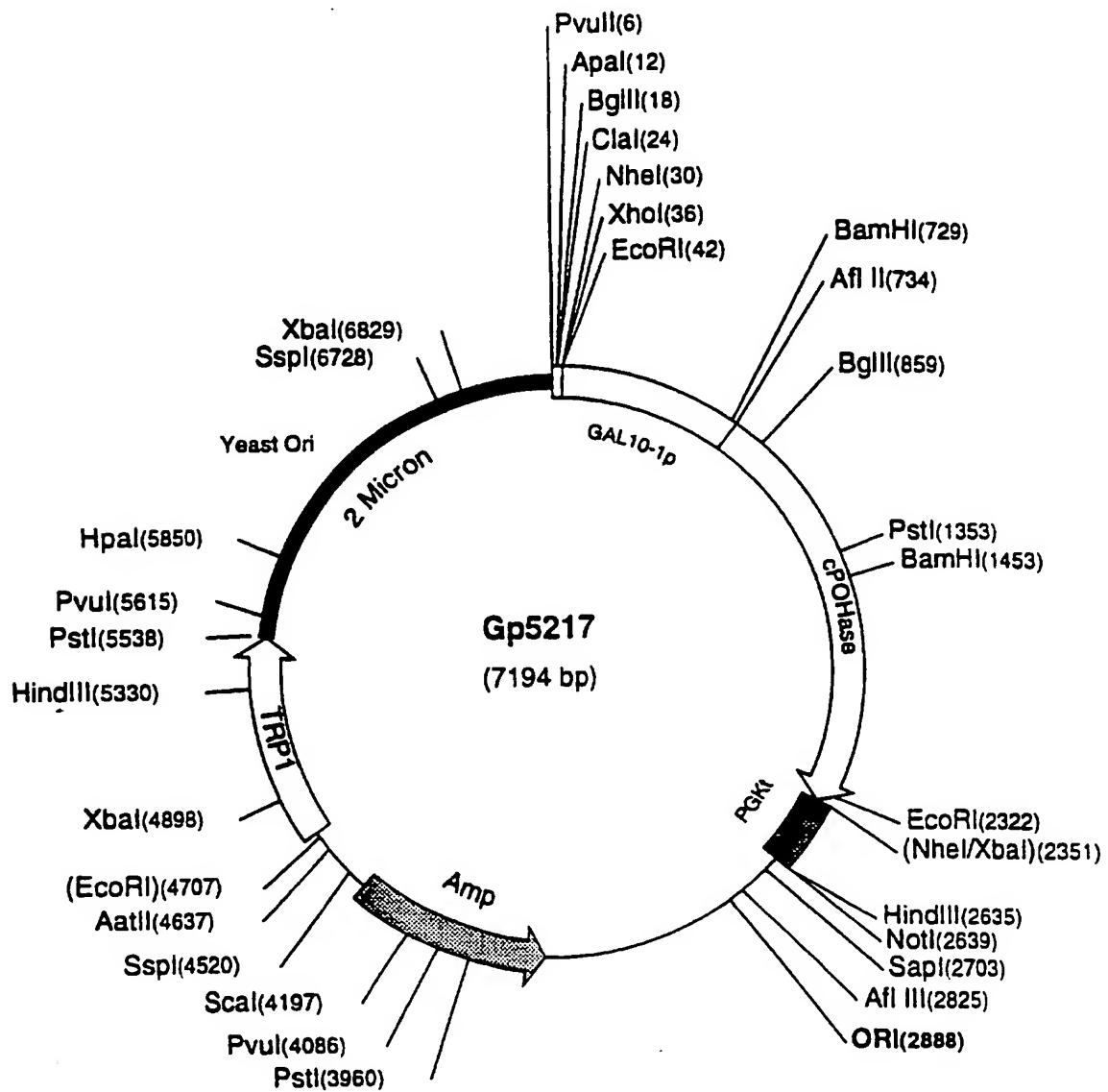


Figure 21

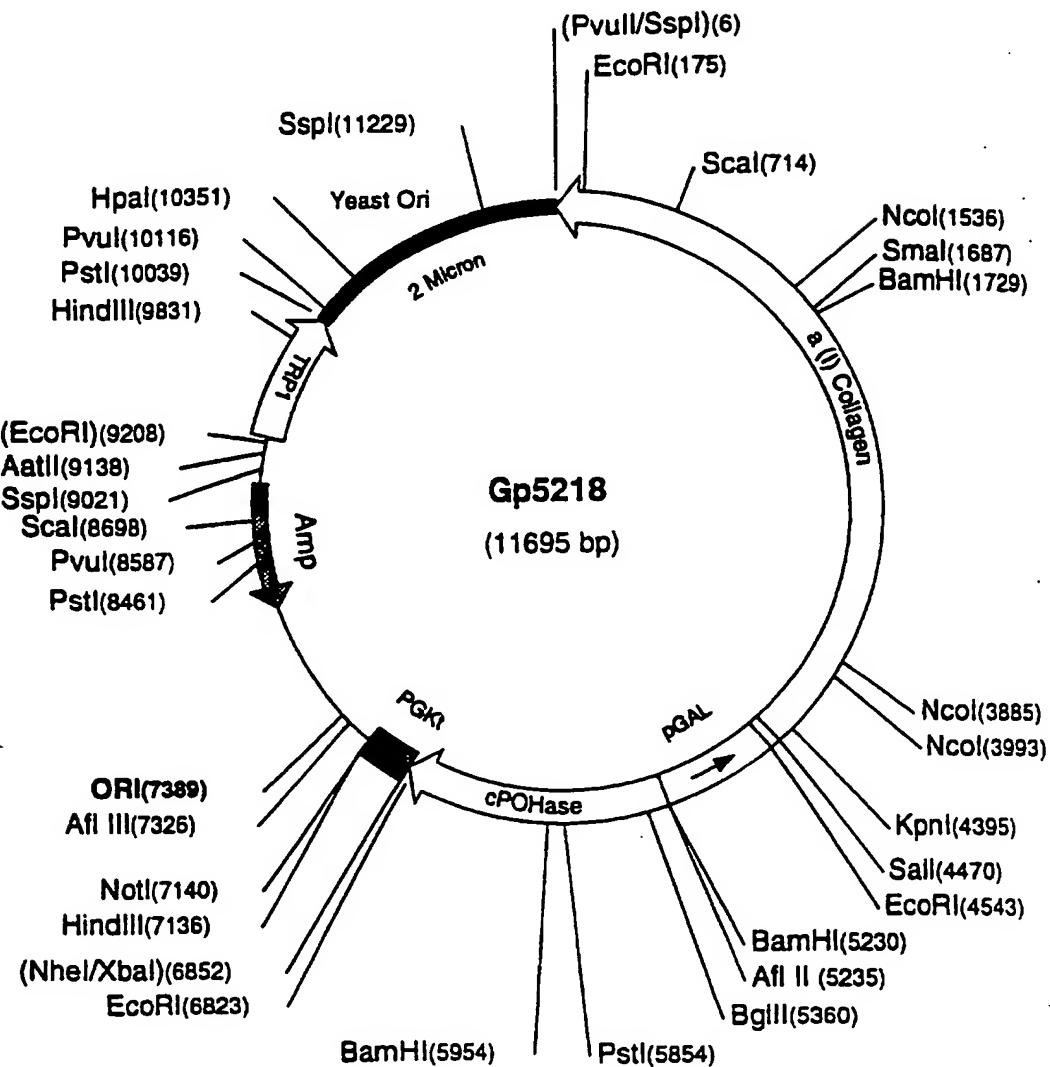


Figure 22

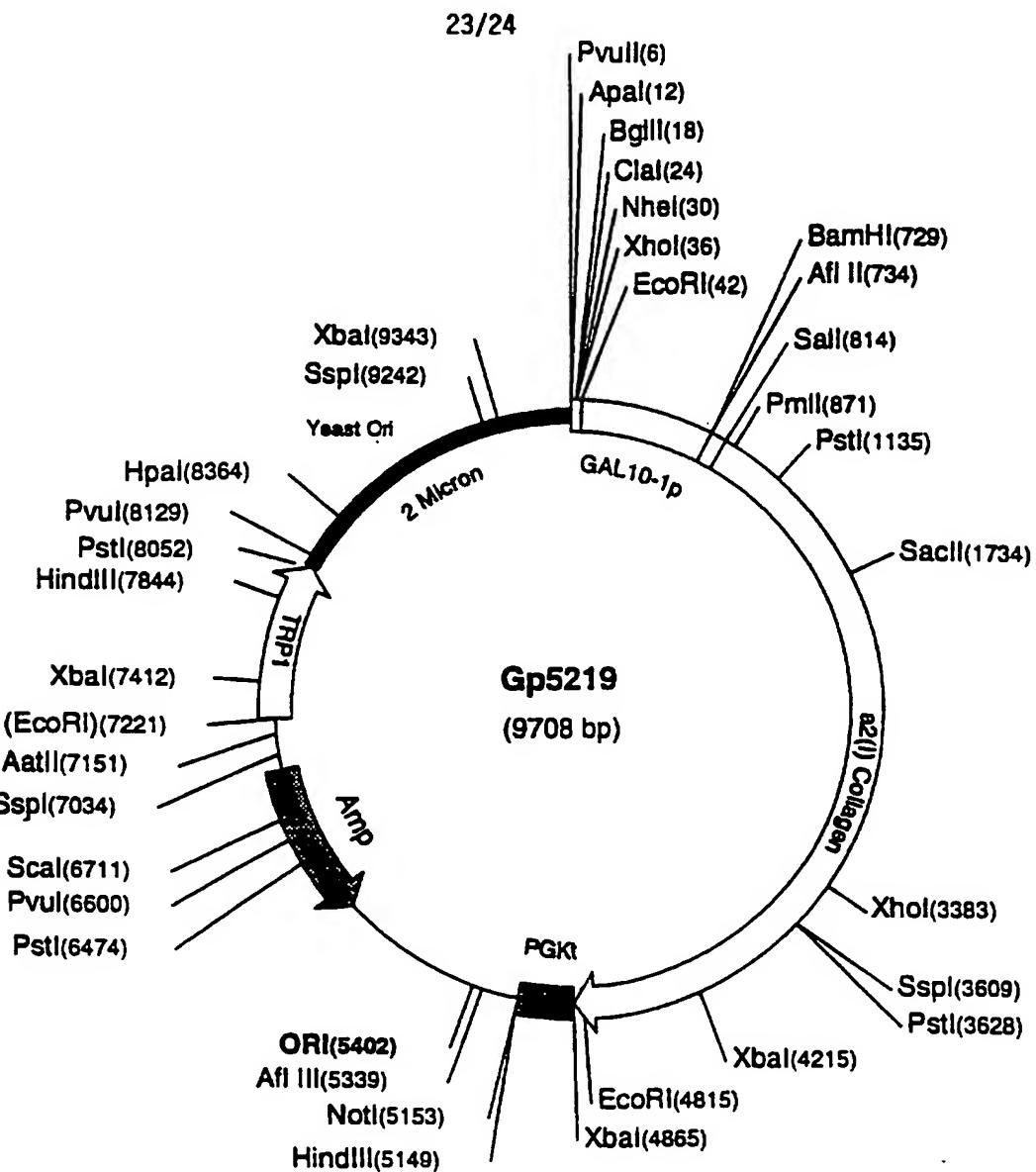


Figure 23

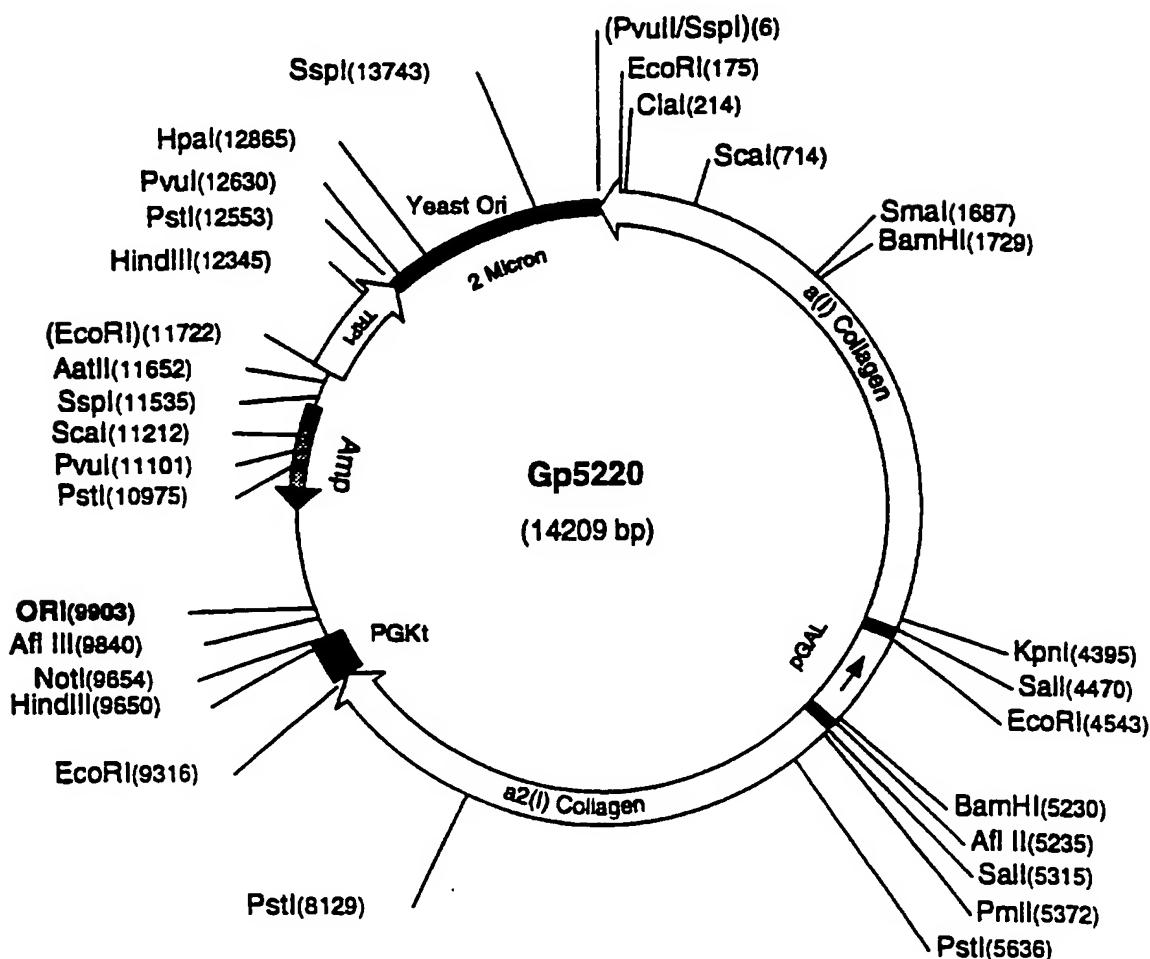


Figure 24

# INTERNATIONAL SEARCH REPORT

Int'l. application No.  
PCT/US96/16646

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/39, 31/01; C07K 1/00; C12N 1/16, 1/18; C12P 21/02  
US CL :530/356; 514/2; 435/254.2, 69.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/356; 514/2; 435/254.2, 69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/07889 A (THOMAS JEFFERSON UNIVERSITY) 29 April 1993 (29-04-93), see entire document, especially pages 1-23.	1-26
Y	ADAMS et al. Collagen Gene Expression. American Journal of Respiratory Cell and Molecular Biology. 1989, Vol.1, pages 161-168, especially pages 161-166.	1-26
Y	ROBINSON et al. Protein Disulfide Isomerase Overexpression Increases Secretion of Foreign Proteins in <i>Saccharomyces cerevisiae</i> . Bio/technology. 1994, Vol.12, pages 381-384, especially pages 381-383.	1-26

Further documents are listed in the continuation of Box C.  See patent family annex.

Special categories of cited documents:	
*A*	document defining the general state of the art which is not considered to be of particular relevance
*E*	earlier document published on or after the international filing date
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
*O*	document referring to an oral disclosure, use, exhibition or other means
*P*	document published prior to the international filing date but later than the priority date claimed
*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*&*	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 JANUARY 1997	31 JAN 1997

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ENRIQUE D. LONGTON Telephone No. (703) 308-0196
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**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/US96/16646**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HITZEMAN et al. Use of Heterologous and Homologous Signal Sequences for Secretion of Heterologous Proteins from Yeast. Methods in Enzymology. 1990, Vol.185, pages 421-440, especially pages 421-434.	1-26